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**New roles for actin-binding proteins and PIP₂ in
intracellular calcium homeostasis**

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Thesis submitted for
Degree of Doctor of Philosophy

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Abbreviations

1-MA: 1-Methyladenine.

ASW: Artificial seawater.

AR: Acrosome reaction.

ARIS: Acrosome reaction inducing substance.

ARC: Adenosine diphosphate ribosyl cyclase.

BAPTA: glycine, N,N'-[1,2-ethanediylbis (oxy-2,1-phenylene)]bis[N-(carboxymethyl)].

cADPr: Cyclic adenosine diphosphate ribose.

Calcium Green: Fluorescent calcium dye calcium green- 488 conjugated to 10 kD dextran.

CaFSW: Ca^{2+} -free seawater.

CCD: Charge couple devise.

CG: Cortical granules.

cGMP: Cyclic guanosine monophosphate.

CICR: Ca^{2+} -induced Ca^{2+} release.

CH: Calponin-homology domain.

DAG: Diacylglycerol.

Dil: dialkylcarbocyanine.

DMSO: dimethylsulfoxide.

EGTA: Ethylene glycol-bis(2-aminoethylether)-*N*, *N*, *N'*, *N'*- tetraacetic acid.

ER: Endoplasmic reticulum.

FE: Fertilization envelope.

GDP β S: GDP-guanosine-5'-0-2-thiodiphosphate.

GFP-Utr: Green fluorescent protein-tagged calponin homology domain of the actin-binding protein utrophin.

GV: Germinal vesicle.

GVBD: Germinal vesicle breakdown.

IB: Injection buffer.

ICSI: Intracytoplasmic sperm injection.

IgG: Immunoglobulin-G.

InsP₃: Inositol -(1, 4, 5)-trisphosphate.

InsP₃R: Inositol -(1, 4, 5)-trisphosphate receptor.

JAS: Jasplakinolide.

LAT-A: Latrunculin-A.

MPF: Maturation promoting factor.

NADP: Nicotinamide adenine dinucleotide phosphate.

NAADP: Nicotinic acid adenine dinucleotide phosphate.

PI3K: Phosphatidylinositol 3-kinase.

PI: phosphatidylinositol.

PIP₃: Phosphatidylinositol -(3, 4, 5)-trisphosphate.

PH: Pleckstrin homology domain.

PIP₂: Phosphatidylinositol (4, 5)-bisphosphate.

PLC: Phospholipase C.

RFP: Red fluorescent protein.

RFP-PH: Red fluorescent protein-tagged fusion protein containing the pleckstrin homology domain of rat PLC- δ 1.

RF: Relative fluorescence.

R40A-RFP: Pleckstrin homology domain containing one site mutation at Arg 40 tagged to the red fluorescent protein.

RFU: Relative fluorescent units.

RyR: Ryanodine receptor.

SOC: Store operated channel.

SH2: Src homology domain 2.

SW: Seawater.

TRP: Transient receptor potential.

List of publications:

1. Chun J.T., Puppo A., **Vasilev F.**, Gragnaniello G., Garante E., and Santella L. (2010). The biphasic increase of PIP₂ in the fertilized eggs of starfish: new roles in actin polymerization and Ca²⁺ signaling. *PLoS One*. 5(11):e14100.
2. **Vasilev F.**, Chun J.T., Gragnaniello G., Garante E., and Santella L. (2012). Effects of ionomycin on egg activation and early development in starfish. *PLoS One*. 7(6):e39231.
3. Santella L., **Vasilev F.**, and Chun J.T. (2012). Fertilization in echinoderms. *Biochem. Biophys. Res. Commun.* 425(3):588-94.

Abstract

Spatiotemporal increase of the intracellular Ca^{2+} is the most universal way to regulate the function of a eukaryotic cell. Owing to a host of actin-binding proteins and enzymes whose activities are modulated by the local concentration of Ca^{2+} , free Ca^{2+} in cytosol serves as a pivotal second messenger in a variety of cell functions. The rise and fall of intracellular Ca^{2+} wave has been best illustrated in eggs at fertilization. However, the molecular mechanism by which intracellular Ca^{2+} is increased in the fertilized egg is largely unknown despite the discoveries of the distinct Ca^{2+} -mobilizing second messengers in the past 30 years. In this thesis, I have used the starfish oocytes to study how Ca^{2+} signaling can be modulated by the actin cytoskeleton, which is known to be dynamically remodelled during meiotic maturation and fertilization of the egg. The principal issues of my experimental work are: (i) to establish the role of actin-binding proteins and PIP_2 in the regulation of the Ca^{2+} signaling; (ii) to study the effect of the Ca^{2+} -store depletion on Ca^{2+} signaling and on the structure and function of the actin cytoskeleton, and (iii) to study the role of the actin-cytoskeleton in establishing the block to polyspermy. Microinjected into starfish eggs, actin-binding protein gelsolin, function-blocking antibody to depactin, and the PIP_2 -sequestering fusion protein that indirectly alters the actin cytoskeleton, all changed a certain aspect of Ca^{2+} signaling. Depletion of the Ca^{2+} store with ionomycin in turn drastically changed the cortical structure and the actin cytoskeleton of the eggs, eventually leading to a deleterious effect on egg activation and early development. Finally, the alteration of the actin cytoskeleton led to failure to establish a fast and slow block to polyspermy. Taken together, this study indicated that the actin cytoskeleton is an important factor that optimizes the Ca^{2+} response at egg activation and guides monospermic fertilization.

CHAPTER I

Introduction

Starfish as an animal model system

The experimental model mostly used in this work was the starfish oocyte. Starfish oocytes were obtained from three different species: *A. aranciatus* (*A. aranciatus*), *Patiria miniata* (also known as *Asterina miniata*) and *Asterina pectinifera* (*A. pectinifera*). Starfish belong to the phylum *Echinodermata*, class *Stellerioide*, subclass *Asteroidea*.

The body of starfish presents radial symmetry. A fully grown organism of *A. aranciatus* is about 50 cm in diameter, and consists of a central disk and five rays. The oral surface containing the mouth is exposed to the substrate, whereas the aboral surface with madreporite is the entry site of the water-vascular system. The reproductive system of the female animal consists of 10 separate ovaries, two in each ray. During oogenesis, there is an increased protein synthesis, growth and differentiation. The breeding season begins upon the action of an unknown environmental cue that stimulates the radial nerves to produce a neuro-hormone called gonad-stimulating substance (Kanatani et al., 1967). This hormone induces production of a maturation hormone 1-methyladenine (1-MA) in the follicle cells surrounding the oocytes. When 1-MA is distributed to the oocyte surface, it induces meiotic resumption in all the species of starfish (Kanatani et al., 1970). Soon after, the mature eggs deprived of follicle cells are released into the seawater. Starfish reproduce by external fertilization, for which sperm and eggs are released in vicinity and at the same time from gonoducts and gonophores that are located in the base of the each ray of male and female bodies, respectively (Villet et al., 1978).

A. aranciatus lives 2-20 m deep in the Mediterranean sea, buried in the sand. These animals were being captured in the bay of Naples, Italy, during the breeding season from February until May. *Patiria miniata* (*P. miniata*) were captured in California, USA, whereas *A. pectinifera* were from the Mutsu Bay, Japan. Animals were kept in the natural

seawater at 15 °C inside the specialized tanks and maintained by the staffs at the Stazione Zoologica of Naples.

Starfish oocytes are a very useful model system for studying Ca^{2+} signaling, oocyte maturation, fertilization and embryonic development. To obtain the gametes, all it takes is to make a small surgical cut where the gonads are located. Once the dissected gonads are placed in seawater, free oocytes sediment from the cut ends. The gonads provide a large quantity of oocytes that are arrested at the first prophase of the meiotic cycle. These oocytes can be induced to undergo meiotic resumption *in vitro* by addition of the maturation hormone 1-MA. Microinjection in these big cells is relatively easy, and the embryos develop rapidly reaching the 32-cell stage within 5 hours. The eggs and embryos are transparent under microscope, enabling excellent observation of morphological traits.

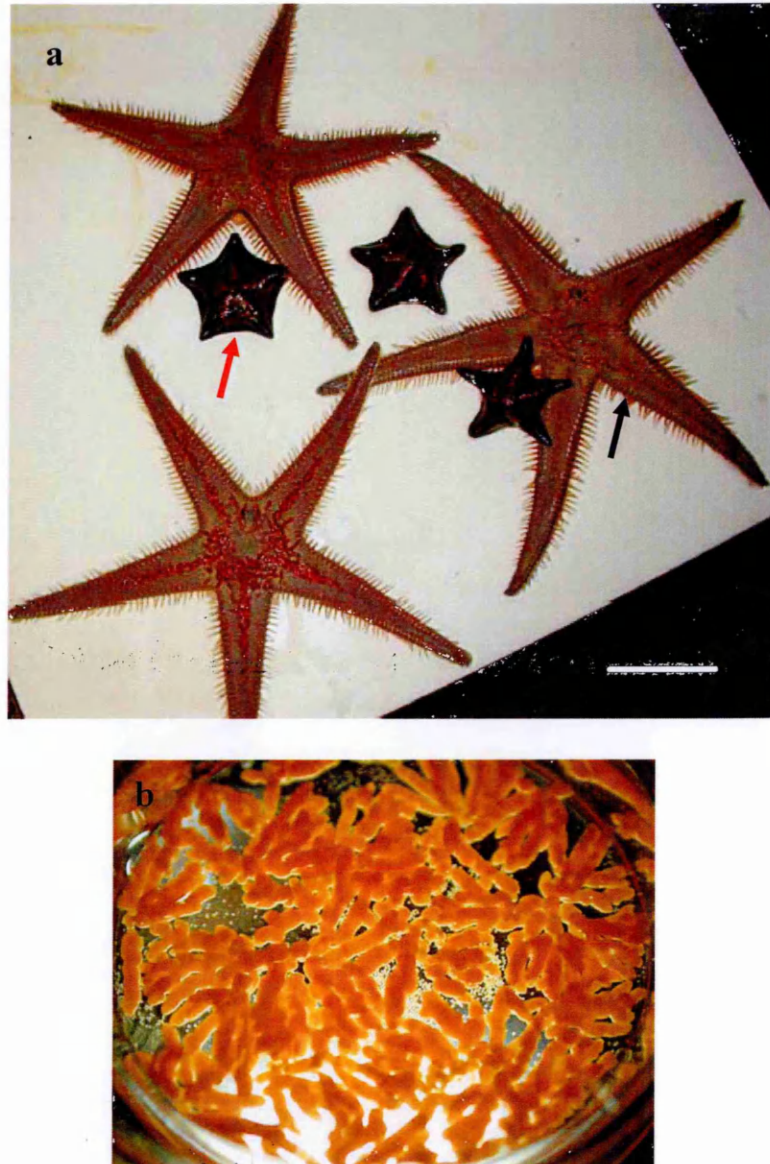


Figure I 1. (a) Adult animal of *A. pectinifera* (red arrow) and *Astropecten araciacus* (black arrow). (b) Female gonads isolated from *A. pectinifera*.

Morphology of starfish oocytes

When the oocytes of *A. aranciacus* and *P. miniata* are fully grown in ovaries, they have a diameter of 300 μm and 190 μm , respectively. These oocytes are characterized by the presence of a big nucleus termed 'germinal vesicle' that can be seen in one side the cytoplasm having a diameter of 70 μm and 60-90 μm , respectively.

The oocytes are surrounded by two layers: a jelly coat and a vitelline layer. The surface of the oocytes emits numerous microvilli 0,3–0,4 μm in length, and the vitelline layer is intimately associated with the oocyte's surface (Shroeder, 1981; Longo et al, 1995). The vitelline layer contains glycoproteins and is about 0,5 μm thick. It is surrounded by the jelly coat which is secreted by the follicle cells (Shroeder, 1981; Santella et al., 1983). The small follicle cells surround the oocytes about 15 μm from the oocytes surface. They make contact with the oocytes surface by means of cytoplasmic extensions that penetrate the jelly layer and the vitelline layer to make contact with the oocytes plasma membrane through desmosome-like attachments. It has been suggested that 1-MA is transmitted from the follicle cells through these processes (Schroeder, 1981).

In the germinal vesicle, the chromatin is decondensed and the nucleolus is visible even under the light microscope. Starfish oocytes are polarised. The germinal vesicle is localised in the animal hemisphere, and the opposite side is defined as the vegetal hemisphere. The germinal vesicle is round in shape, but the region at which it is anchored to the cortex near the plasma membrane is highly convoluted. This region between the plasma membrane and the germinal vesicle is the place where centrosomes and pre-meiotic aster are located (Myazaki et al., 2000). The endoplasmic reticulum (ER) is evenly distributed in the cytoplasm (Jaffe and Terasaki, 1994) together with other intracellular organelles, like lipid droplets and yolk granules (referred also as yolk bodies). Oocytes are also abundant with cortical granules, ribosomes, Golgi complexes and mitochondria. Yolk granules occupy about a half of the cytoplasm volume of the oocytes. They are 1-2 μm in diameter and are the major sites of nutrient storage. The majority of the proteins that

are contained in the yolk granules are not required for early embryogenesis in starfish, as their depletion is not significant until the larval stage (Reimer and Crawford, 1995).

A large assemblage in the cortex of the oocyte is the subplasmalemmal cytoskeleton (Sardet et al., 2002). The tightly packed actin filaments are absent only where the germinal vesicle is connected to the plasma membrane. Through this 'corridor,' the polar bodies are extruded. Whereas the yolk granules are dispersed in the entire cytoplasm, the cortical granules are localized in the cortex of the oocytes (Reamer and Crawford, 1995). Cortical granules have diameter of about 1 μm and display an irregular ovoidal shape, and contain sulphated acid mucopolysaccharides and proteins (Sousa and Azevedo, 1989). Cortical granules are positioned 1-3 μm away from the plasma membrane in immature oocytes. During meiotic maturation, they translocate to the surface area immediately underneath the plasma membrane. There are about 40,000-50,000 of cortical granules in starfish oocytes of *Pisaster ochraceus* and *A. pectinifera* (Schroeder, 1985). It has been suggested that they are important during fertilization and at embryonic development. Their major function is to form the fertilization envelope following their exocytosis. The fertilization envelope serves as a physical barrier to block polyspermy (Vacquier et al., 1973; Schuel, 1978). During embryonic development, they secrete extracellular matrix components (Teimer and Crawford, 1995). Besides the cortical granules, additional class of large vesicles called 'acidic vesicles' reside in the cortex of the oocytes. These vesicles ranging 1-2 μm in diameter are seen to be exocytosed during the vitelline layer elevation at fertilization (Vasilev et al., 2012).

Stimulation of the cell cycle resumption by 1-Methyladenine

Fully grown oocytes isolated during the breeding season are arrested in the first prophase of the meiosis. At this stage, the oocytes are not able to be successfully fertilized by one sperm. The oocytes can be induced to resume the meiotic cycle *in vitro* by adding the maturation hormone 1-MA. The hormone provokes two successive M-phases of the cell cycle, bringing the oocyte to the pronucleus stage (Kishimoto, 2003).

In response to the maturation hormone the immature starfish oocytes resume the meiotic cycle and undergo nuclear disassembly during a process referred to as 'germinal vesicle breakdown' (GVBD). The time course for the GVBD is different for the various species, being 30 minutes for *P. miniata* and 60 minutes for *A. aranciacus*. It takes several minutes since the sharp outline of the germinal vesicle becomes irregular and finally disappears until the complete intermixing of the nuclear and cytoplasmic contents.

The dose of 1-MA necessary for inducing the oocytes to undergo meiotic maturation *in vitro* is from 1 to 10 μM . The 'hormone dependent period' is defined as the minimal time needed for the oocytes to be exposed to the hormone in order to display clear signs of maturation in 50% of the oocytes (Nemoto, 1982). It is highly dependent on temperature (increases as the temperature is lowered) and the 1-MA concentration (Schuetz 1969a).

How 1-MA stimulates the oocytes has not been fully understood so far. However, it was shown that the hormone works only when it is added to the oocytes from outside. Oocytes microinjected with the hormone does not undergo meiosis, suggesting that a cell surface receptor is involved in the process. In line with this, when the uptake of the hormone into the cell was blocked it could still elicit its biological activity (Doree and Guerrier, 1975), suggesting that its effect is mediated by a signal transduction pathway. The receptor for 1-MA has not been identified yet (Shida and Shida, 1976). Indeed, the response of oocytes to 1-MA is significantly impeded by Triton X-100 that extracts proteins from the plasma membrane (Morisawa and Kanatani, 1978).

It has been suggested that 1-MA could restart meiosis through a receptor-coupled heterotrimeric G-proteins (Shilling et al., 1989). The α , β and γ subunits of the G-protein has been purified from the plasma membrane of starfish oocytes (Tadenuma et al., 1991; Tadenuma et al., 1992), and the effect of 1-MA has been shown to be sensitive to the pertussis toxin (PTX) (Shilling et al., 1989). The inhibition of the 1-MA-induced maturation by microinjection of the pertussis toxin (Shilling et al., 1989) and the $G_{i\alpha}$ supports that the 1-MA effect is mediated by the receptor linked with the G-protein. In support of the idea, microinjection of $G_{\beta\gamma}$ into the starfish oocytes led to GVBD in the absence of 1-MA (Jaffet et al., 1993; Chiba et al., 1993). The effectors of the $G_{\beta\gamma}$ subunits are suggested to be located in the cytoplasm of the oocytes, since injection in the central region of the cytoplasm close to the germinal vesicle induces faster GVBD than injection near the plasma membrane (Chiba et al., 1993). It has been reported that the target for the G-protein $\beta\gamma$ subunits can be phosphatidylinositol 3-kinase (PI3K) (Lopez-Illasaca et al., 1997; Stephens et al., 2002). PI3K has been proposed to be downstream effector of $G_{\beta\gamma}$ because the inhibition of PI3K by wortmannin and LY294002 blocks the 1-MA-induced meiosis and MPF activation (Sadler and Ruderman, 1998).

The finding that the cytoplasmic extract taken from the hormone-stimulated frog oocytes can stimulate the progression of the cell cycle in non-stimulated cells (Masui and Markert, 1971) suggested the presence of a cytoplasmic factor sufficient for inducing maturation. Similar results were obtained from the starfish oocytes microinjected with the cytosol of 1-MA-treated oocytes. These oocytes underwent GVBD and polar body formation without 1-MA (Kishimoto and Kanatni, 1976). The Maturation-Promoting Factor (MPF) has been shown to be the underlying factor in the 1-MA-dependent oocyte maturation, and is made of a complex of two proteins: cyclin B and Cdc2 kinase (cyclin B/Cdc2). The signaling pathway leading to the activation of the MPF has been elucidated in several experimental systems. 1-MA indirectly activates the kinase Akt (protein kinase B, PKB), that phosphorylates and down-regulates Myt1, a membrane-associated kinase

that in turn phosphorylates Cdc2 on tyrosine 15 and threonine 14 (Mueller et al., 1995; Okumura et al., 2002). The phosphorylation of the Cdc2 keeps the complex cyclinB/Cdc2 in an inactive state. The activity of Cdc25, a tyrosine phosphatase that activates MPF by dephosphorylating the tyrosine residues, is regulated by autophosphorylation and dephosphorylation and is sensitive to protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Masui, 2001; Prigent and Hunt, 2004). MPF is inactive in the cytoplasm of immature starfish oocytes, but accumulates in the nucleus after being activated in the cytoplasm (Ookata et al., 1992). The translocation of MPF and Cdc25 is thought to be essential for the final MPF activation. In starfish, MPF enters the oocyte nucleus at the animal hemisphere (Terasaki et al., 2003). The disassembly of the nuclear lamina occurs as a result of a phosphorylation of lamins by cyclinB/Cdc2 (Peter et al., 1990). In addition, phosphorylation by cyclinB/Cdc2 controls the spindle assembly and directs interaction of MPF with microtubules (Ookata et al., 1993) and centrosomes (Perez-Mongiovi et al., 2000). Cdc2 apparently phosphorylates many proteins implicated in the meiotic process, and our laboratory has found that cytoskeletal elements such as actin filaments might also be modulated by MPF (Lim et al., 2003).

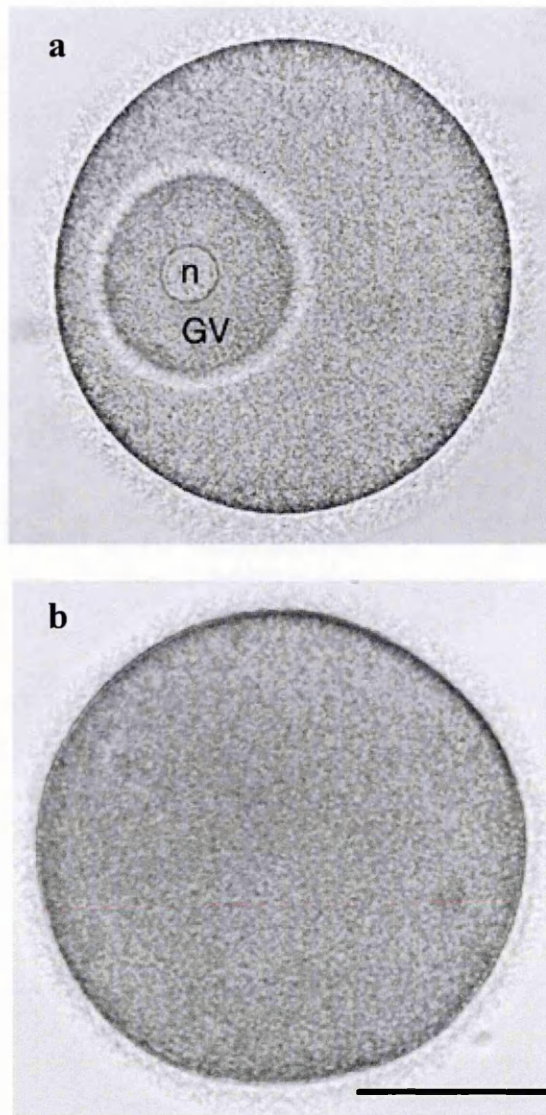


Figure I 2. (a) Immature oocyte of *A. aranciacus* arrested at the first prophase of meiosis. (b) An oocyte tuned into a mature egg after 70 minutes incubation with 1-MA. Abbreviation: n, nucleolus; GV, germinal vesicle. Scale bar, 100 μm . (Modified from Santella et al., 2012).

Morphological changes during meiotic maturation

Within a few minutes after the meiotic cycle is resumed, the first visible change take place in the oocyte surface. The follicle cells surrounding the oocytes migrate at one side as the desmosome-like attachments connecting the follicle cells to the oocytes disappear. After 1-2 minutes' exposure to 1-MA, the actin-filled cytoplasmic projections (spikes) may appear and persist for 20 minutes during the hormonal treatment, as the actin cytoskeleton is polymerized and subsequently depolymerised (Schroeder and Stricker, 1983). In addition, starfish oocytes have relatively long microfilaments-filled microvilli that emanate from the cell surface into the vitelline layer. They diminish in length and decrease in density following 1-MA treatment. Hence, in the first several minutes after meiotic resumption, the actin cytoskeleton undergoes extensive reorganization.

During meiotic maturation, the mechanical properties of the starfish oocytes change, by showing a decrease in rigidity of the cell by the time of GVBD (Nakamura and Hiramoto, 1978; Nemoto et al., 1980). After the induction of the maturation, the surface tension of the oocytes decreases rapidly and remains low until the extrusion of the first polar body. A sharp rise and fall in tension is again observed during the formation of the second polar body (Hiramoto, 1964, 1976; Nakamura and Hiramoto, 1978; Shoji et al., 1978). The changes of the surface tension of the oocytes appear to be a result of the intermixture of nucleoplasm and cytoplasm because it does not occur in the fragmented oocytes lacking the nucleus (Yamamoto and Yoneda, 1983; Yamamoto 1985). As the changes in the actin cytoskeleton induced by cytochalasin-B leads to a dramatic decrease in stiffness of starfish oocytes (Nemoto et al., 1980), it was suggested that the F-actin organization is responsible for the stiffness of the oocytes (Heil-Chapdelaine and Otto, 1996).

In parallel, the electric properties of the plasma membrane changes during meiotic maturation. The initial resting potential marks -80 mV in the starfish oocytes, but the

membrane potential shifts to a stable level of -10 to -20 mV at the time of GVDB (Dale et al., 1979; Miyazaki and Hirai, 1979).

The cortical granules (CG) are scattered in the periphery of the cortical region of the oocytes (Longo et al., 1995). During meiotic maturation, they translocate to the egg surface and attach themselves to the plasma membrane. With their long axis often perpendicular to the plasma membrane, they form a monolayer (Longo et al., 1995). This process depends on the actin cytoskeleton (Santella et al., 1999, Wessel et al., 2002). In mature eggs, the cortical granules positioned in the subplasmalemmal region are ready to be exocytosed in response to the sperm-induced cytosolic Ca^{2+} increase or to the Ca^{2+} -liberating second messengers, e.g. inositol-1, 4, 5- trisphosphate (InsP_3), cyclic-ADP ribose (cADPr) and nicotinic acid adenine dinucleotide phosphate (NAADP) (Picard et al., 1985; Dargie et al., 1990; Lee and Aarhus 1995). This suggests that the triggering event for the CG exocytosis is the intracellular Ca^{2+} increase. It was estimated that about 96% of the egg's CG undergo exocytosis when Ca^{2+} is mobilized by fertilizing sperm or ionophore, (Longo et al., 1995).

During meiotic maturation, there is a significant reorganization of the ER. The structural changes in the ER membranes have been studied by using the fluorescent lipophilic carbocyanine dye: dialkylcarbocyanine (DiI). The dye emits mild fluorescence when is not bound to the target, but becomes bright when it binds to the cell membranes. Once bound to the biological membrane it begins to spread laterally and stains the whole leaflet of the membrane. Microinjection of the oil drop saturated with DiI into the starfish oocytes labels the cisternae of ER that are uniformly distributed throughout the cytoplasm. In mature eggs, the ER acquires the form of spherical shells surrounding yolk granules that appear to be interconnected (Jaffe and Terasaki, 1994). During this period of meiotic maturation, the dynamic changes of the actin cytoskeleton are also accelerated. The study was performed to examine the possible effect of the actin cytoskeleton on the GVBD phase of the meiotic cycle (Stricker and Shatten, 1991). It was shown that on the onset of the

GVBD and the pre-GVBD changes in the nuclear shape has been affected by the drugs that affect the actin microfilaments, but not the ones that disrupt the microtubules. Hence, the actin cytoskeleton is implicated in the meiotic events in starfish oocytes.

Calcium homeostasis

The functioning of the multicellular organisms is based on exchange of various signals among cells to modulate and coordinate their activity. During evolution, they have developed a complex ways of generating and processing information. Calcium is the second messenger that has great versatility and controls many cellular processes (e.g. fertilization, muscle contraction, secretion, gene transcription, apoptosis) (Carafoli, 2002). The intracellular Ca^{2+} signaling usually starts with external stimuli (neurotransmitters, hormones, growth factors) and sperm that interact with the receptors on the cell surface to trigger synthesis of diffusible factors (second messengers) inside the cells. They in turn bind to a ligand-gated Ca^{2+} channels and thereby release Ca^{2+} from the internal stores. On the other hand, the external stimuli can also induce Ca^{2+} influx from the extracellular space (Berridge, 1993). The concentration of the free Ca^{2+} outside the cells is orders of magnitude higher than inside: mM vs. nM. The Ca^{2+} does not diffuse freely across the plasma membrane, and the low levels inside the cells are thus maintained by allowing only a certain amount of Ca^{2+} to enter through carefully regulated ion channels. The free Ca^{2+} concentration in the physiological conditions are kept at the 100 nM in cytosol, but it can rise to 1-10 μM within seconds after the stimuli. Several factors contribute to the maintenance of the cytosolic Ca^{2+} concentration. Ca^{2+} forms low affinity complexes with small compounds such as phosphates, ATP, amino acids, and acidic phospholipid. Ca^{2+} can also form high affinity complexes by reversibly binding to the specific sites in the Ca^{2+} -binding proteins (Carafoli et al., 2001). These molecules serve as a primary buffer for Ca^{2+} in cytosol and effectively provide a mechanism by which the local concentration of the intracellular Ca^{2+} changes rapidly. Intracellular Ca^{2+} concentration is largely regulated by the release-absorption cycle at the Ca^{2+} stores. Cytosolic Ca^{2+} can be increased by the release from the intracellular stores or by Ca^{2+} influx from the extracellular media, while the surplus Ca^{2+} can be removed by the action of the intracellular Ca^{2+} pumps and by sequestration with the Ca^{2+} -binding proteins and exchangers. The energy-consuming action

of the Ca^{2+} pumps thus restores the resting level of the cytosolic Ca^{2+} by transporting the ions into the lumen of the organelles (e.g. ER, nuclear envelope, Golgi apparatus, and mitochondria) and to the extracellular space. Hence, the cytoplasmic Ca^{2+} concentration is maintained by the tightly regulated interplay of Ca^{2+} release and Ca^{2+} uptake.

In eukaryotic cells, the Ca^{2+} channels residing on the plasma membrane are mainly gated by the changes of the membrane potential, by the binding of the ligands, or by the emptying of the intracellular Ca^{2+} stores. The Ca^{2+} increase inside the cells can be achieved by the ligand-gated InsP_3 and cADPR residing mainly on the membranes of the ER. Such ligands are second messengers, and several different substances have been shown to induce a rapid Ca^{2+} release from the internal stores. InsP_3 is so far the best characterized second messenger that releases Ca^{2+} by gating the InsP_3 receptors (InsP_3R) from the ER (Streb et al., 1983), but also from non-reticular Ca^{2+} stores, e.g., the Golgi apparatus (Pinton et al., 1998), lysosomes (Haller et al., 1996; Scrinivas et al., 2002), secretory granules (Gerasimenko et al., 1996; Quesada et al., 2001) and nuclear envelope (Gerasimenko et al., 1995; Hennager et al., 1995; Santella and Kyoizuka, 1997). cADPr is another second messenger shown to mediate the intracellular Ca^{2+} release from the ER, whereas NAADP has been shown to release Ca^{2+} from stores distinct from ER, and the acidic vesicles were proposed to be the possible Ca^{2+} store.

InsP_3 -mediated intracellular Ca^{2+} increase

The history of InsP_3 dates back to 1953 when it was found that the interaction of the acetylcholine with muscarinic receptor on the plasma membrane of pancreas cells stimulated the incorporation of [^{32}P] phosphate into the minor phospholipid phosphatidylinositol (Hokin and Hokin, 1953). Then, 30 year later, Michael Berridge and colleagues identified in pancreatic cells the InsP_3 as a soluble product of the turnover of inositol lipids to induce Ca^{2+} increase (Streb et al., 1983). Besides the InsP_3 , the turnover of inositol phospholipids led to a production of diacylglycerol (DAG), which remains in the plasma

membrane. DAG also functions as an intracellular messenger by activating protein kinase C (Takai et al., 1979; Kishimoto et al., 1980). Since then, InsP_3 was tested and proven to be a universal second messenger to release Ca^{2+} in a vast number of cell types, including those of fungi, animals and plants (Berridge, 2007).

InsP_3 is produced by hydrolysis of its precursor, the plasma membrane phosphatidylinositol 4, 5-bisphosphate (PIP_2) by the phospholipase C. Then, InsP_3 diffuses in the cytoplasm and binds to InsP_3R on the membranes of the ER to elicit Ca^{2+} release. In mammals, three types of InsP_3R have been identified ($\text{InsP}_3\text{R1}$, $\text{InsP}_3\text{R2}$ and $\text{InsP}_3\text{R3}$) (Furuichi et al., 1989; Furuichi et al., 1994). The amino acid sequence of the receptor protein can be divided in three domains, a short C-terminal hydrophobic domain that contains the membrane spanning sector, the large regulatory domain in the centre, and the flexible N-terminal domain that projects into the cytoplasm and is responsible for binding of InsP_3 (Mikoshiha, 1997). The receptor was found in the membrane of the nucleus (Nicotera et al., 1990), and may be also responsible for the Ca^{2+} release from secretory vesicles (Petersen, 1996) and Golgi apparatus (Pinton et al., 1998). Besides InsP_3 , the channel activity of InsP_3R can be regulated by the Ca^{2+} , ATP, and by its phosphorylation/dephosphorylation status (Berridge, 1995).

The phosphoinositide family consists of seven derivatives of phosphatidyl-inositol (PI) that are formed by phosphorylation at the 3-, 4- and 5-position of the inositol ring (Bout and Divecha, 2009). PIP_2 can be synthesized by the action of two distinct phosphoinositide kinases. Phosphatidylinositol 5-phosphate 4-kinase (PIP4K) phosphorylates phosphatidylinositol 5-phosphate at the 4-position, whereas phosphatidylinositol 4-phosphate 5-kinase (PIP5K) phosphorylates phosphatidylinositol 4-phosphate at the 5-position to form PIP_2 . The level of PIP_2 is tightly regulated by the aforementioned kinases and phosphomonoesterases that dephosphorylate PIP_2 to PI(4)P and PIs (Bout and Divecha, 2009).

PI and its phosphoinositide derivatives constitute ~10% of the total cellular lipids in most cells. PIP₂ is enriched in the inner leaflet of the plasma membrane and is estimated to be the most abundant phosphorylated derivative of PI. It may comprise from 0,3-1,5 % of the phospholipids at the plasma membrane of mammalian erythrocytes, lymphocytes and hepatocytes (Saarikangas et al., 2010). The presence of PIP₂ and phosphatidylinositol 3, 4, 5-triphosphate (PIP₃) in the plasma membrane controls many important reactions such as generation of intracellular second messengers, exocytosis, endocytosis, and reorganization of the actin cytoskeleton (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006; Odorizzi et al., 2000). Interaction of PIP₂ with various proteins at the membrane can induce formation of microdomains of PIP₂ clusters with specific proteins (e.g., actin-binding proteins MARCKS, ezrin, dynamin, MIM) that may regulate membrane trafficking and receptor signaling (Saarikangas et al., 2010). The presence of different PIs species and phosphoinositide-binding actin-binding proteins in the nucleus suggest that phosphoinositides could regulate the poorly understood dynamics of nuclear actin (Irvine, 2003; Vartiainen, 2008). Extra-plasma membrane pool of PIs has been shown to be enriched in early endosomes and multivesicular bodies (Gillooly et al., 2000; Gillooly et al., 2003), as well as in the Golgi apparatus, in late endosomes, and lysosomes (D'Angelo et al., 2008; Michell et al., 2006).

The hydrolysis of PIP₂ to produce InsP₃ and DAG is carried out by the phospholipase C (PLC) (Rhee, 2001). The InsP₃ mediated Ca²⁺ release can be activated by many G protein-coupled receptors and tyrosine kinase-linked receptors. There are multiple isozymes of the PLC that are grouped in at least six subfamilies (β , γ , δ , ϵ , ζ and η) (Saunders et al., 2002; Nakahara et al., 2005). PLC β , γ , and δ have been identified in the sea urchin eggs (De Nadai et al., 1998) and PLC β and γ have been identified in those of starfish (Runft et al., 2004). The structure of the PLC contains combinations of various regulatory domains that include the pleckstrin homology (PH) domain, the Src homology 2 (SH2) domain, EF-hand motifs and C2 domain. The active site of the enzyme is composed

of the X and Y domains, which associate to form the PIP₂ cleavage site. The PH domain has been proposed to tether the enzyme to the plasma membrane and thereby keep it associated with the lipid surface facilitating its access to the substrate, PIP₂. It appears that the PH domain from different isozymes of PLC can interact with PIP₂ and PIP₃, and with the $\beta\gamma$ subunits of heterotrimeric G proteins (Camps et al., 1992). On the other hand, the SH2 domain interacts with the tyrosine kinases (Noh et al., 1995; Weiss, 1993). In starfish eggs, the two PLC γ -SH2 domains have been shown to be responsible for the InsP₃-mediated intracellular Ca²⁺ release at fertilization (Runft et al., 2004).

Cyclic ADP ribose-mediated intracellular Ca²⁺ increase

Ryanodine receptors (RyR) are the other Ca²⁺-releasing channels on the membranes of the ER. It was initially described in the sarcoplasmic reticulum of skeletal and cardiac muscle (Fleischer et al., 1985), but now is known to be present in the non-muscle cells as well. The name of the receptor derives from the name of the plant alkaloid ryanodine that cause contraction of the skeletal muscle (Jenden and Fairhurst, 1969). Then, the receptor was isolated and purified and showed to possess the Ca²⁺ channel activity in the lipid bilayer membranes (Lai et al., 1988).

In the sea urchin homogenates, cADPr was identified as the second messenger to release Ca²⁺ from the RyR (Clapper et al., 1987). cADPr is the low molecular weight metabolite of the pyridine nucleotide nicotinamide adenine dinucleotide (β -NAD⁺) synthesised by the ADP-ribosyl cyclase (ARCs) (Lee, 1999). In sea urchin eggs the ARC isoforms are found to be present and active inside acidic and exocytotic vesicles, where their substrate (β -NAD⁺) is transported and the cADPr produced (Davis et al., 2008). cADPr has been reported to be a second messenger to play a role in the sperm-initiated Ca²⁺ signaling at fertilization of sea urchin and starfish eggs (Kuroda et al., 2001; Nusco et al., 2002; Leckie et al., 2003). Injection of cADPr into the sea urchin eggs induces a Ca²⁺ increase similar to the response at fertilization, formation of the fertilization envelope and

DNA synthesis (Dargie et al., 1990). In contrast, blocking the Ca^{2+} releasing mechanism of cADPr alters the pattern of Ca^{2+} signaling at fertilization (Galione et al., 1993; Lee et al., 1993; Leckie et al., 2003). In starfish eggs, the cADPr has been suggested to be implicated rather in the propagation of the sperm-induced Ca^{2+} signals than in their initiation (Moccia et al., 2006). However, the mechanism by which cADPr induces intracellular Ca^{2+} increase is not yet fully established. As Ca^{2+} -induced Ca^{2+} release (CICR) is mediated by the RyR and InsP_3R , nanomolar concentrations of cADPr can greatly increase the sensitivity of the CICR mechanism to Ca^{2+} at RyR (Galione et al., 1991; Lee, 1993). In addition, cADPr can activate Ca^{2+} influx in some cell types (as neutrophils, pancreatic β -cells) that can occur via the stimulation of the transient receptor potential cation channel, subfamily M, member 2 (TRPM2) in the plasma membrane (Partida-Sanchez et al., 2001; Togashi et al., 2006).

Intracellular Ca^{2+} increase stimulated by NAADP

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the newly discovered second messenger that induces a Ca^{2+} increase from microsomes isolated from sea urchin eggs (Lee and Aarhus, 1995). NAADP, a derivative of nicotinamide adenine dinucleotide phosphate (NADP), is produced by the same enzyme that produces cADPr: ADP-ribosyl cyclase. The enzyme catalyzes the conversion of the amide nicotinamide group of the common metabolite NADP to a carboxyl group. One example of the ARC is the mammalian CD38 that was shown to catalyze NAADP hydrolysis as well.

The target channel for NAADP has been shown to be distinct from those already known. NAADP-dependent Ca^{2+} increase was insensitive to inhibitors of the InsP_3 -, cADPr- and CICR-based intracellular Ca^{2+} release mechanisms (Clapper et al., 1987). The only way to block it is to self-desensitize it by using high concentrations of NAADP (Aarhus et al., 1996; Genazzani et al., 1996). By fractionation of the sea urchin eggs homogenates, it was shown that NAADP targets the Ca^{2+} stores that are distinct from the ER. The NAADP-responsive organelles were found on the bottom of the Percoll density

gradients, well separated from the cADPr-sensitive ER that was on the top of the gradient (Lee, 1996). Then, by *in situ* stratification of live sea urchin eggs, it was conclusively shown that the NAADP-responsive stores were located at the pole of the stratified sea urchin eggs (where the organelles are) that was separate and opposite to that of the cADPr- and InsP₃-sensitive stores (Lee and Aarhus, 2000). These organelles were further identified as the reserve granules, a lysosome-related organelles in the sea urchin eggs (Churchill et al., 2002), and the same finding was also confirmed in mammalian cells (Kinnear et al., 2004). The NAADP receptor was further suggested to reside on the membrane of the acidic organelles, since the NAADP-evoked Ca²⁺ release was inhibited by the agents that disrupt Ca²⁺ storage in acidic organelles, e.g., proton pump inhibitor bafilomycin and the lysomotropic agent glycyl-L-phenylalanine-2-naphthylamide (GPN) (Churchill et al., 2002). Recently, the voltage-gated two-pore channels (TPCs) have been proposed as the candidate for the NAADP receptors to be expressed in the lysosomal and endosomal membranes (Calcraft et al., 2009).

Additional information on the NAADP-induced Ca²⁺-releasing mechanism has been obtained by using starfish eggs. NAADP was shown to cause a Ca²⁺ release selectively in the subplasmalemmal region of the starfish eggs (cortical flash). This response is dependent upon the presence of external calcium. This led to the discovery of the inwardly directed Ca²⁺ current gated by NAADP (Moccia et al., 2003). Then, it was suggested that the NAADP-evoked Ca²⁺ increase could be mediated by the store operated Ca²⁺ type channel (SOC) because the emptying of the internal stores by thapsigargin, promoted the same influx of Ca²⁺. Finally, NAADP has been proposed to be the initiator of the intracellular Ca²⁺ increase initiated by the sperm that is further amplified by cADPr and InsP₃ during fertilization of starfish eggs (Moccia et al., 2006).

Ca²⁺ signaling during meiotic maturation

The importance of the intracellular Ca^{2+} signaling in resumption of the meiotic cycle has been controversial. The first report that the maturation hormone 1-MA induces intracellular Ca^{2+} increase in starfish oocytes came from the studies on *Marthasterias glacialis* oocytes (Moreay et al., 1978). These oocytes responded with the Ca^{2+} increase in the absence of external Ca^{2+} , indicating that the released Ca^{2+} came from the internal stores. However, in the oocytes of other species such as *Asterias forbesi* and *Asterina miniata*, no Ca^{2+} increase was detected after stimulation with 1-MA (Doree et al., 1990). Furthermore, artificial increase of intracellular Ca^{2+} by use of InsP_3 microinjection did not resume the meiotic cycle in the absence of 1-MA (Picard et al., 1985). Conversely, blocking the InsP_3R by the specific peptide (InsP_3 -sponge) was shown not to affect the 1-MA-induced Ca^{2+} response (Iwasaki et al., 2002). Whether the Ca^{2+} increase by 1-MA plays a role in the oocytes meiotic maturation was tested by the use of the Ca^{2+} buffering agents BAPTA and EGTA. Buffering the cytoplasmic Ca^{2+} by microinjection of these agents did not inhibit the 1-MA-induced meiotic resumption in oocytes of *Asterina miniata* (Witchel and Steinhardt, 1990). On the other hand, the microinjection of BAPTA directly into the nucleus prevented the dissolution of the nuclear envelope in the oocytes of starfish *A. pectinifera* (Santella and Kyozyuka, 1994). Interestingly, photoliberation of the preinjected caged InsP_3 and caged cADPr in the nucleus stimulated GVBD in the 50 % of the treated oocytes (Santella and Kyozyuka, 1997).

The 1-MA-mediated Ca^{2+} increase was demonstrated to initiate always at the vegetal hemisphere of the starfish oocytes and to be restricted to the subplasmalemmal regions (Kyozyuka et al., 2008). Structural reorganization of the actin cytoskeleton in the subplasmalemmal region was further shown to be implicated in the 1-MA-induced Ca^{2+} release. The spatiotemporal pattern of the 1-MA-induced Ca^{2+} release was shown to be significantly affected by various agents promoting actin cytoskeleton assembly or disassembly, e.g. latrunculin-A (LAT-A), heparin, jasplakinolide (JAS) and G-proteins inhibitors (Kyozyuka et al., 2008; Kyozyuka et al., 2009). The actin cytoskeleton

reorganization by the preinjection of the actin-modulating protein cofilin into the cytoplasm of the starfish oocytes was also shown to affect the 1-MA-induced cortical Ca^{2+} release by enhancing the Ca^{2+} release by two-fold (Nusco et al., 2006).

During meiotic maturation, starfish oocytes become more responsive to the stimuli that induce an increase of intracellular Ca^{2+} , e.g. InsP_3 , NAADP, cADPr and sperm (Chiba et al., 1990; Nusco et al., 2002; Santella et al., 2000). Microinjection of the same amount of InsP_3 evokes higher Ca^{2+} release in mature eggs compare to immature oocytes. Indeed, the longer the oocytes are exposed to 1-MA, the higher the Ca^{2+} response becomes (Lim et al., 2003). While in mammalian oocytes this is in part due to the increased expression and redistribution of cortical InsP_3R (Fujiwara et al., 1993), the same does not hold for starfish oocytes. The higher Ca^{2+} response to InsP_3 in mature eggs of starfish is neither due to the increased expression and redistribution of the InsP_3R (Iwasaki et al., 2002), nor due to the increased amount of Ca^{2+} storage in the ER (Chiba et al., 1990; Lim et al., 2003). In starfish oocytes, the apparent sensitization to InsP_3 was rather linked to the state of the actin cytoskeleton. When the maturation process was induced by 1-MA in the presence of the actin-depolymerizing drug LAT-A, the Ca^{2+} release in response to InsP_3 was strongly inhibited (Lim et al., 2003). Surprisingly, the treatment of mature *A. aranciacus* eggs with LAT-A induced both Ca^{2+} influx and the release from the internal stores 10 minutes after incubation (Lim et al., 2002). The massive Ca^{2+} release induced by LAT-A was observed in mature eggs, but not in oocytes. As the increased sensibility to InsP_3 in mature eggs is paralleled with the morphological changes in the ER, it is noteworthy that the actin cytoskeleton is dynamically rearranged in response to 1-MA before the ER reorganization that peaks at the time of GVDB (Jaffe and Terasaki, 1994). It has been shown that the redistribution of the ER is dependent on microfilaments (FitzHarris et al., 2007) as well as microtubules (Whitaker, 2006). Finally, the increased sensitivity to InsP_3 was shown to be delayed by 30 minutes in starfish oocytes preincubated with roscovitine, a specific inhibitor of MPF (Lim et al., 2003). At variance with other animal species (Machaca et al.,

2007), MPF does not phosphorylate InsP₃R in starfish oocytes (Lim et al., 2003). Inhibition of the LAT-A-induced Ca²⁺ release by roscovitine indicated that the sensitization might be dependent on rearrangement of the actin cytoskeleton modulated by MPF (Lim et al., 2003).

During meiotic maturation, the cADPr- and NAADP-dependent Ca²⁺ release mechanisms are also sensitized. Photoactivation of injected cADPr in starfish oocytes induces multiple patches of Ca²⁺ release in the cortical region and the signal then spreads from these initial spots to the entire cell. In mature eggs, photoactivation of the caged cADPr induces Ca²⁺ increase from one or two sites, which is followed by the cortical flash and further propagation of the Ca²⁺ wave in the entire egg (Nusco et al., 2006). The NAADP-induced Ca²⁺ release is higher in starfish eggs compared to immature oocytes, and the Ca²⁺ signal in eggs unlike to that in oocytes is dependent on the external Ca²⁺. Moreover, the NAADP-induced Ca²⁺ response in mature eggs is more sensitive to the L-type inhibitors than in the immature oocytes. Hence, the NAADP in mature eggs was suggested to work on the calcium channel at the plasma membrane (Santella et al., 2000).

Ca²⁺ signaling at fertilization

The sperm-egg interaction begins with a series of morphological and physiological events, involving recognition, adhesion and fusion between gametes. The most common event that occurs inside the fertilized eggs in all studied species, from marine invertebrates to mammals, is a massive Ca²⁺ release inside the fertilized eggs. The fertilized egg is then activated, and the zygote starts to develop into the embryo (Ciapa and Chiri, 2000). The intracellular Ca²⁺ increase starts from the site of sperm interaction and traverses the egg cytoplasm as a wave. Depending on species, the Ca²⁺ increase may occur as a single transient (sea urchin, starfish) or as repetitive oscillations (mouse, hamster, bovine, rat, human) (Ciapa and Chiri, 2000; Santella et al., 2004).

Fertilization in marine invertebrates usually occurs outside the body, and thus requires an extremely sensitive mechanism of chemotaxis between the eggs and the proper sperm (Santella and Chun, 2011). Recognition and interaction of the molecules residing on the sperm surface and in the egg jelly coat is the initial step of the species-specific fertilization that further leads to mutual activation of the sperm and egg. When the sperm encounter the egg jelly, the species-specific egg jelly components trigger the acrosome reaction (AR) in the sperm. In echinoderms, it is a Ca^{2+} -based exocytosis of the sperm's acrosomal vesicles, which contains the digestive enzymes that pave the way for the fertilizing sperm. During this process, the head of the sperm extends a long process (acrosomal process) filled with actin filaments. The three components of the egg jelly known to trigger AR in starfish are ARIS (AR-inducing substance), co-ARIS, and asterosap (Hoshi et al., 2000). ARIS is a high molecular weight sulphated glucoprotein, which requires a diffusible cofactor, steroid saponin co-ARIS. In sea urchin and starfish, asterosap works as a chemotactic agent that stimulates the guanylate cyclase (asterosap receptor) located on the sperm flagellar plasma membrane that stimulates the motility of the sperm (Hoshi et al., 2000; Matsumoto et al., 2008).

Even though Ca^{2+} has been known to be the trigger of egg activation for several decades, much is yet to be known about the upstream signaling pathway and the nature of the Ca^{2+} release from the internal stores. Three major hypotheses have been proposed on how the fertilizing sperm initiates the massive Ca^{2+} release inside the eggs. The first model is based on the idea that the sperm introduces a bolus of Ca^{2+} during gamete fusion (Jaffe 1983, 1991), which was later modified to the suggestion that channels in the surface membrane of the sperm allowed it to act as a conduit for Ca^{2+} entry (Creton and Jaffe, 2001). Ca^{2+} entered in this way would further act to trigger CICR inside the egg (Jaffe, 1991). Despite the finding that the sperm-egg fusion in both sea urchin and mice always precedes the Ca^{2+} release in the egg (McCulloh and Chambers, 1992; Lawrence et al., 1997), a number of studies have cast a doubt on the validity of this model, as direct

injection of Ca^{2+} into sea urchin, Ascidian, or mammalian eggs has all failed to induce further Ca^{2+} release (Whitaker and Swann, 1993; Swann and Ozil, 1994). Moreover, no elevation of the cytoplasmic Ca^{2+} concentration occurs at the moments of gametes fusion in mouse (Jones et al., 1998).

The second model proposed is based on the idea that the sperm introduces a soluble factor into the egg at fertilization. Microinjection of sperm extracts has been shown to induce cytosolic Ca^{2+} increase in eggs of sea urchins (Dale et al., 1985), marine worms (Stricker, 1997), ascidians (Kyoizuka et al., 1998) and mammalian species (Swann, 1990; Wu et al., 1997), mimicking the Ca^{2+} signals at fertilization. The sperm-released putative factors in sea urchins were initially thought to be InsP_3 and NAADP (Whitaker and Irwine, 1984; Churchill et al., 2003), whereas in mouse eggs a soluble protein such as oscillin was proposed as a sperm factor that diffuses in the egg cytoplasm after the gametes fusion (Parrington et al., 1996). More recently, the novel sperm-specific PLC, the $\text{PLC}\zeta$ (Saaunders et al., 2002) was proposed as a potent sperm factor that serves as a physiological agent of egg activation in mammals (Swann et al., 2006).

The third hypothesis suggests that the ligand on the surface of the sperm interacts with the egg surface receptor that would further trigger InsP_3 production via PLC activation. The activation of PLC could be mediated by receptor-bound G proteins. This model is supported by the analyses of the timing of the cortical granules breakdown in sea urchin eggs upon microinjection of InsP_3 and by the consideration that the inhibitor of G proteins, GDP-guanosine-5'-0-2-thiodiphosphate ($\text{GDP}\beta\text{S}$), prevents the sperm-induced eggs activation (Turner et al., 1986; Mohri and Hamaguchi, 1989). However, the action of $\text{GDP}\beta\text{S}$ has not been verified as it may have non-specific inhibitory effect at high concentration (Crossley et al., 1991). Another slightly different version of the receptor-mediated egg activation model suggests involvement of the tyrosine kinase pathway that activates the γ isoform of PLC. The $\text{PLC}\gamma$ is activated when its two Src homology-2 (SH2) domains interact with tyrosine kinases. There are several lines of evidence suggesting that

a Src-like kinase mediates the activation of the PLC γ in eggs of starfish and other animals. Activation of PLC in this pathway may produce InsP₃ and subsequent Ca²⁺ increase during egg activation at fertilization (Runft et al., 2002; Townley et al., 2006). Microinjection of starfish egg with human Src protein evokes Ca²⁺ release and DNA synthesis (Giusti et al., 2000). Recombinant proteins containing the two SH2 domains of PLC γ that specifically inhibit PLC γ , but not PLC β , completely blocked Ca²⁺ release at fertilization, suggesting that the Ca²⁺ release was due to the activation of PLC γ and InsP₃ formation (Carroll et al., 1997). Hence, while the central role for starfish egg activation has been ascribed to PLC γ , the putative receptor remains to be identified.

For a long time, the initial increase of the cytosolic Ca²⁺ at fertilization has been considered to derive mainly in response to the synthesis of InsP₃ (Runft., 2002; Whitaker, 2006; Miyazaki, 2007; Ducibella and Fissore, 2008). Early studies with sea urchin observed a substantial increase of polyphosphoinositide in fertilized eggs (Turner et al., 1984), and the generation of InsP₃ coincided with the Ca²⁺ wave (Ciapa et al., 1992). In line with the idea that the InsP₃ plays a critical role in developing the Ca²⁺ response at fertilization mouse eggs of which InsP₃R were blocked by specific antibodies failed to display the sperm-activated repetitive Ca²⁺ spikes (Miyazaki et al., 1992). Similar negation of the Ca²⁺ signaling at fertilization in mice was observed upon ablation of the PLC ζ (Nomikos et al., 2005), suggesting the indispensable role of InsP₃ in the process. However, at variance with the mammals, the role of the InsP₃ at fertilization is not conclusively established in echinoderms. Experiments on sea urchin eggs did not show a temporal coincidence between the major InsP₃ increase and the generation of the Ca²⁺ wave. Rather, the onset of the Ca²⁺ increase coincided with the elevation of the second messenger cGMP but preceded the increase of cADPr and InsP₃, as the major rise of InsP₃ takes place only after the Ca²⁺ has already initiated (Kuroda et al., 2001). In addition, when the changes of the plasma membrane PIP₂ level was monitored in living cells on a real-time basis by the use of the RFP-tagged PH domain of PLC- δ 1, the plasma membrane PIP₂ level of the

starfish eggs began to decline 10 seconds after the Ca^{2+} wave was initiated by the fertilizing sperm (Chun et al., 2010). Hence, these results cast a doubt on the role of InsP_3 as a very initial trigger of the Ca^{2+} response in echinoderm eggs at fertilization.

Although cGMP and cADPr induce intracellular Ca^{2+} increase when injected into eggs of many species in which RyR is present, the contribution of the cADPr to the Ca^{2+} release at fertilization has been established only in sea urchin eggs. cADPr is generated after the initiation of the Ca^{2+} wave in fertilized eggs of sea urchins to sustain the duration of the Ca^{2+} transient (Leckie et al., 2003). On the other hand, blocking RyRs with 8- NH_2 cADPr inhibiting the cADPr-sensitive Ca^{2+} release had no effect on the Ca^{2+} signaling in starfish eggs at fertilization (Nusco et al., 2002). Likewise, the inhibition of the RyRs by ruthenium red or large dose of ryanodine (Gerasimenko and Gerasimenko, 2004) did not impair the development of the Ca^{2+} wave (Moccia et al., 2006).

The third second messenger that may release Ca^{2+} at fertilization of echinoderm eggs is the NAADP. Photoactivation of NAADP in starfish eggs induces the Ca^{2+} increase as a cortical flash which then propagates centripetally as a wave (Lim et al., 2001; Santella, 2005). This NAADP-evoked cortical flash that closely resembles the one induced by the sperm was shown to be completely inhibited by the L-type Ca^{2+} channel blockers, implying that NAADP may induce the Ca^{2+} influx from seawater through a voltage-sensitive ion channel. As the NAADP response was also blocked by SOC inhibitors, it was suggested that NAADP might work through a TRP-like channel. In addition, as NAADP can trigger the membrane depolarization leading to the initial cortical Ca^{2+} increase mimicking the effect of the fertilizing sperm, it was suggested that the NAADP may mediate the initial phase of the Ca^{2+} signaling at fertilization (Moccia et al., 2006). NAADP generates Ca^{2+} influx in sea urchin eggs as well, and it was suggested that NAADP might be delivered from the fertilizing sperm (Churchill et al., 2003) as the activated sperm possess high concentrations of the NAADP (Billington et al., 2002). Desensitization of the NAADP receptors with high dose of NAADP prevented the onset of the fertilization potential in

starfish eggs (Moccia et al., 2006), and reduced the cortical flash and the Ca^{2+} waves in sea urchin eggs (Churchill et al., 2003). However, the identity and the nature of the NAADP-sensitive Ca^{2+} stores are not fully established. Whereas lysosome-like acidic organelles were identified as NAADP-sensitive Ca^{2+} stores in sea urchin eggs (Churchill et al., 2002), the NAADP-evoked membrane depolarization was not affected by the drugs interfering with acidic organelles nor by the agents known (Galione and Petersen, 2005) to block the action of NAADP on RyRs (Moccia et al 2006).

Fast block and slow block to polyspermy

In majority cases of fertilization in vivo, only one sperm enters the egg at fertilization. In echinoderm eggs, the elevation of the fertilization envelope is thought to be useful in blocking the entry of the supernumerary sperm (Schuel, 1985), but this process takes considerable amounts of time before it produces a visible mechanical barrier against the supernumerary sperm (slow and permanent block). Thus, it was predicted that a fast mechanism may exist to ensure monospermic fertilization (Just, 1919). Indeed, it was estimated that the rate of refertilization in sea urchin eggs is reduced by a factor of 20 (Rothschild and Swann, 1950). In 1976, Jaffe suggested that the fast block to polyspermy in sea urchin eggs may be mediated by electrical changes in the membrane potential (Jaffe 1976). Like neurons and muscle cells, oocytes and eggs are electrically excitable. It has long been known that echinoderm oocytes can fire action potentials in response to stimuli (Tyler et al., 1956). In sea urchin eggs, the resting membrane potential of the egg quickly depolarizes upon fertilization. This rapid change of the membrane potential consists of three distinct phases. The first response can take the form of a small 'step depolarization' as was seen in the eggs with the low resting potentials (about -30 mV), or fire action potentials in the eggs with high resting potential (about -80 mV). In both cases, the initial electric response is followed by a sustained depolarization that is termed 'activation potential' or 'fertilization potential', which usually remains at positive levels for more than

5 minutes until it begins to decline back to the resting potential in 10 minutes (Dale et al., 1978; Hinkley et al., 1986; McCulloch and Chambers, 1992). In *Strongilocentrotus purpuratus* (*S. purpuratus*) the activation potential is attained within the 3 seconds after the apparent sperm attachment, a timing that is expected as a plausible mechanism for fast block to polyspermy. The fast electrical block to polyspermy was supported by several other studies in which sperm entry was prevented by holding the membrane potentials at positive values in the eggs of sea urchins, frogs and marine worms *Urechis caupo* (Gould-Somero et al., 1979; Cross and Elinson, 1980; Lynn and Chambers, 1984). However, no fast block in *S. purpuratus* eggs was obtained by other experimenters (Byrd and Collins, 1975), and the existence of the fast block in echinoderms has remained controversial (Dale and Monroy, 1981). When the sperm-egg interaction is interrupted by addition of warm seawater containing detergent 20 seconds after fertilization, successful re-fertilization took place in the non-fertilized zones, indicating that the block to polyspermy is more linked to the local changes in the egg cortex (Allen and Hagström, 1955). Another evidence arguing against the idea of the fast electrical block to polyspermy was obtained by artificially holding the sea urchin eggs at negative values of the membrane potential that appeared to produce monospermic fertilization, and not polyspermy (Dale and De Felice, 2010).

Physiological and morphological studies have suggested that immature oocytes of sea urchin are more receptive than mature eggs towards sperm. Not having the cortical granules positioned beneath the plasma membrane, immature oocytes do not undergo cortical granules exocytosis and therefore become fertilized by 10-15 sperm, although the first sperm induces potential of 50 mV in amplitude (Dale and Santella, 1985). This result points to the importance of the cortical granules in promoting the monospermic fertilization, and suggests a potential existence of a limited number of interacting sites on the oocyte surface that may become less receptive during meiotic maturation.

Actin-binding proteins and PIP₂ in regulation of the intracellular Ca²⁺ homeostasis

Actin is a 42 kDa protein that is highly abundant and strictly conserved in all eukaryotic cells. Actin filaments together with microtubules and the intermediate filaments constitute the cytoskeleton. It is implicated in the regulation of various cell functions such as cell motility, intracellular transport, mechanical support, secretion, phagocytosis, cytokinesis, while the nuclear actin may regulate gene expression and transcription regulation (Gieni et al., 2009).

The central dogma in the intracellular Ca²⁺ signaling is that the Ca²⁺-releasing second messengers act on the corresponding ion channels to release Ca²⁺ from the lumen of the internal stores (such as ER) in response to various stimuli. While it is well known that the intracellular increase of Ca²⁺ induces changes in the actin cytoskeleton (Forscher, 1989), little is known about whether and how the changes in the actin cytoskeleton dynamics can affect the intracellular Ca²⁺ signaling. In starfish oocytes, the actin cytoskeleton may play a role in optimizing the InsP₃-mediated Ca²⁺ release during meiotic maturation (Lim et al., 2003). LAT-A also induces intracellular Ca²⁺ release in mature eggs which is characterized by a cortical flash, cytoplasmic propagation and oscillatory liberation (Lim et al., 2002). The Ca²⁺ increase by LAT-A occurs in the absence of the external Ca²⁺ indicating that the active rearrangement of the actin cytoskeleton stimulates Ca²⁺ release from the internal stores. Moreover, inducing changes in the cortical actin cytoskeleton of starfish oocytes by the use of several pharmacological agents such as JAS, LAT-A and heparin not only resulted in a blockade of the 1-MA induced Ca²⁺ increase (Kyojuka et al., 2008) but also significantly altered the patterns of the sperm-induced Ca²⁺ signals (Puppo et al., 2008). When more physiological tools were used to modify the state of the actin inside the starfish oocytes, e.g., the actin-modulating protein cofilin, the Ca²⁺ signals was shown to be significantly increased in response to various second messengers (e.g. InsP₃, NAADP), maturation hormone and the sperm (Nusco et al., 2006). Finally, altering the cortical actin cytoskeleton in starfish eggs by masking the plasma membrane

PIP₂ with the PH-domain of the PLC- δ delayed the Ca²⁺ response in response to the sperm and the Ca²⁺-releasing second messenger InsP₃ (Chun et al., 2010).

Inside the cells, actin can be present as a monomer (G-actin), or in a polymerized form constituting a long filament (F-actin). The latter constantly undergoes a dynamic process of treadmilling. Approximately half of the actin pool in living cells exists in a monomeric form (Forscher, 1989), and the equilibrium between F-actin and G-actin is achieved largely due to the action of an array of actin-binding proteins such as profilin, (Wolven et al., 2000), destrin (Ono, 2007), and gelsolin (Sun et al., 2007). This class of proteins regulates the state of the actin cytoskeleton by severing, capping, twisting or cross-linking of the actin filaments (Dos Remedios et al., 2003). The activity of the actin-binding proteins to control the state of the actin can be strongly regulated by the PIP₂ (Lassing and Lindberg, 1985; Janmey and Stossel, 1987; Sehi and Wehland, 2000; Yin and Janmey, 2003).

As was aforementioned, the PIP₂ is one of the key molecules in intracellular Ca²⁺ signaling, since it serves as a substrate for the PLC to produce the Ca²⁺-releasing second messenger InsP₃ (Rhee, 2001). Besides, PIP₂ itself may be considered a second messenger that has many cellular functions (McLaughlin et al., 2002; Di Paolo and De Camilli, 2006). By interacting with the actin-binding proteins (Hilpela et al., 2004), PIP₂ can modulate their activity and location inside the cell, and could induce local changes in the actin cytoskeleton through those proteins that regulate the actin assembly or disassembly (Sechi and Wehland, 2000; Yin and Janmey, 2003). On the other hand, once bound to the actin-binding proteins, e.g. profilin (Witke, 2004), the effective level of PIP₂ can be locally and temporally modulated to limit its availability to PLC. Hence, the actin-binding proteins can regulate the cytoskeletal dynamics and modulate the intracellular Ca²⁺ signaling. The Ca²⁺ in turn could induce changes in the actin cytoskeleton by affecting the Ca²⁺-dependent actin-binding proteins, e.g. gelsolin.

Actin filaments could modulate the cytosolic concentration of free Ca^{2+} by modulating the efficacy of the intracellular Ca^{2+} channels either directly or through the secondary changes in the structure of the ER. In this context, the ER reorganization during meiotic maturation and fertilization may be in part due to the actin cytoskeletal changes. If it induces significant changes in the curvature of the membrane, it might also cause a 'stretch-induced' activation of the ion channels on the ER (Jaffe and Tersakai, 1994; Jaffe, 2007, Chun and Santella, 2009). The direct link between the actin filaments and the InsP_3R was also demonstrated in mouse cell to regulate the activity and the subcellular distribution of the receptor (Turvey et al., 2005). Hence, by altering the structure of the actin cytoskeleton, it is conceivable that cells can either inhibit or enhance Ca^{2+} release from the internal stores. The regulatory role of the actin filaments has been shown in both the Ca^{2+} influx and the ligand-gated Ca^{2+} release through ER (Rosado et al., 2000; Furuyashiki et al., 2002; Sabala et al., 2002; Wang et al., 2002).

Finally, a more radical hypothesis has been suggested for the actin to be responsible for the Ca^{2+} storage and release during the polymerization and depolymerization of the actin filaments (Lange, 1999). Actin molecules bind Ca^{2+} and other divalent cations with high affinity (Kasai and Oosawa, 1968, 1969). According to this theory, Ca^{2+} bound in F-actin become practically inaccessible to external ions for exchange, as the retention time of Ca^{2+} is increased by up to 3000 times increased. In this way, the F-actin may work as a potential Ca^{2+} store (Lange, 1999). On the other hand, the Ca^{2+} bound monomers are ready to release Ca^{2+} in exchange of Mg^{2+} that is 1,000 times more concentrated in cytosol. Thus, depolymerisation of the Ca^{2+} -charged microfilaments may serve as a mechanism to release Ca^{2+} in certain subcellular loci. Indeed, disruption of Ca^{2+} -loaded actin filaments by ultrasonic treatment or by incubation with profilin can produce release of Ca^{2+} *in vitro* (Lange and Brandt, 1996; Lange, 1999). In favour of this hypothesis the strongest evidence so far came from starfish eggs that were able to release Ca^{2+} during the treatment with the

actin depolymerising drug LAT-A in the absence of any other Ca^{2+} releasing agent (Lim et al., 2002).

CHAPTER II

Materials and methods

Oocyte preparation

Starfish *A. aranciatus* were collected from the Bay of Naples during the breeding season from January to May and were kept in the tanks with circulating cold natural seawater (15 °C) until July. The female gonads from the animals were dissected out by making cut on the arms close to the central disc. By gentle shaking of the female gonads, the immature oocytes (containing large germinal vesicle) were released in the filtered natural seawater (FSW) or in the artificial seawater (ASW: 490 mM NaCl, 8 mM KCl, 10 mM CaCl₂, 12 mM MgCl₂, 2.5 mM NaHCO₃, pH 8.0), 30 minutes before starting the experiments. Certain experiments were performed in the Ca²⁺-free seawater (500 mM NaCl, 8 mM KCl, 12 mM MgCl₂, 2.5 mM NaHCO₃, 2 mM EGTA, pH 8.0). Immature oocytes that had spontaneously undergone meiotic maturation (germinal vesicle breakdown) were discarded.

Starfish *P. miniata* (also known as *Asterina miniata*) and *A. pectinifera* have been collected from the California coast, USA and from Mutsu Bay, Japan, respectively. The animals were kept at 15°C in tanks filled with the seawater from the Tyrrhenian Sea and diluted with distilled water in a proportion of 9:1.

Sea urchin animals of *Paracentrotus lividus* (*P. lividus*) were captured in the Bay of Naples and kept at 15°C in tanks with circulating natural seawater. The breeding season begins in January and ends in summer. The induction of spawning in laboratory is achieved by injecting KCL (0,5M) into the animal.

Meiotic maturation was stimulated by the addition of the maturation hormone 1-methyladenine (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 µM for *P. miniata* and *A. pectinifera* oocytes and 10 µM for *Astropecten auranciatus* oocytes. Sea urchin eggs are already meiotically matured after the dissection of the gonads. All experiments were performed at 18 °C.

Male gametes were extracted dry and kept at 4 °C. For fertilization experiments 2 µl of dry sperm were suspended in 1 ml of natural seawater and 20 µl of this suspension was added to the 1ml of mature eggs to obtain the final sperm dilution 1:25000.

Microinjection

Microinjection of the oocytes was performed by air pressure (regulated by the Transjector 5246, Eppendorf) through borosilicate glass capillaries (O.D: 1.0 mm, I.D. 0.58 mm, Sutter Instrument, Novato, CA) prepared with the PN-30 puller (Narishige, Japan). The final concentration of the injected substances inside the cells is up to 50-100 times lower than their concentration in the injecting pipette and the volume of the injected material is estimated to be 1 % of the volume of the cell.

Ca²⁺ imaging

All Ca²⁺ imaging experiments were performed by use of epifluorescence microscopy. Fluorescent calcium dye Calcium Green-conjugated to 10 kD dextran (Calcium Green) (Invitrogen, Eugene, Oregon, USA) was suspended in the injection buffer (10 mM Hepes, 100 mM L-Asp at 7.0 pH) (IB) and microinjected (5 mg/ml, pipette contraction) into the cells. In the case when the Ca²⁺ measurements was performed as a result of a photolysis of caged InsP₃, only Calcium Green was microinjected before doing the experiment. In all other experiments, the oocytes were microinjected with the mixture of the calcium dye (Calcium Green) (5 mg/ml) and Rhodamine Red (5 mg/ml) (Molecular Probes), in order to eliminate the artificial signals emerging due to the movement or changes in the volume of the cell during the acquisition (see in Data processing). Changes in the cytosolic Ca²⁺ levels were detected by using a cooled CCD camera (MicroMax, Princeton Instruments, Inc., Trenton, NJ) mounted on a Zeiss Axiovert 200 microscope with a Plan-Neofluar 20x/0.50 objective. The excitation and emission filter wheels (Sutter

Instruments, Co., Novato, CA) were controlled using a computer controlled shutter (Lambda 10-2, Sutter Instruments).

Photolysis of caged InsP₃

Immature oocytes were microinjected with a mixture of Calcium Green (5 mg/ml) and caged InsP₃ (5 μ M, pipette concentration, Molecular Probes) and kept for 5-10 minutes for the substances to diffuse inside the cells. To activate the caged InsP₃ (Molecular Probes) microinjected eggs were mounted on the CCD camera and irradiated with 330 nm UV light by using the computer controlled shutter system (Lambda 10-2, Sutter Instruments). The duration of the UV irradiation was 15 seconds in the experiments with the depactin-antibody during meiotic maturation, and 32 seconds in the experiments with the calcium ionophore-ionomycin.

Confocal microscopy

The starfish oocytes and eggs microinjected with fluorescent probes were viewed with two laser-scanning confocal microscopes. One is the Zeiss LSM 510 META Laser Scanning Confocal Microscope (Jena, Germany) with excitation at 488/520 nm and emission at 500/555 and 560 nm. A Plan-Neofluar 25x/0.80 objective water lens was used to produce optical slices from the specimens. The second one is the Olympus Fluoview 200, equipped with the UplanApo 20x/0.70, 40x/0.85 and 60x/1.20 (W) objectives, and used with the laser powered to the 50 %. The images were captured as digital computer files and examination of the fluorescence was performed using MetaMorph image analysis software.

Light and Transmission electron microscopy (TEM)

Starfish oocytes and eggs were fixed together with proper control samples in filtered seawater containing 1 % glutaraldehyde (pH 8.0) for 1 h at room temperature and

then post-fixed with 1 % osmium tetroxide for 1 h. Specimens were dehydrated in increasing concentrations of alcohol and embedded in EPON 812. The polymerized resin containing the fixed material was sectioned in two ways. For the experiments following ionomycin and 1-MA treatment the semi-thin (1 micron) sections were stained with toluidine blue and examined by light microscopy with a Zeiss Axiovert 200 microscope, and the images were captured by the CCD camera. For the TEM analysis of the samples from the experiments done by using ionomycin and other substances, the ultrathin sections were stained with 2 % uranyl acetate and 0.2 % lead citrate, and examined with a LEO 912 AB energy filter transmission electron microscope.

Staining of F-actin in living cells

Immature oocytes and mature eggs of starfish were microinjected with the Alexa Fluor 488- or 568-conjugated phalloidin (50 μ M, pipette concentration) (Invitrogen, Eugene, Oregon, USA) diluted in methanol. After each microinjection, the oocytes or eggs were incubated in the natural seawater for 10 minutes and then mounted on an experiment-chamber bathed in seawater and observed either by the laser-scanning microscope, or by a CCD camera. The F-actin was also visualized by microinjection of the GFP-tagged calponin homology domain of the actin-binding protein of human utrophin (GFP-Utr). In this case the protein was suspended in injection buffer and after each microinjection the cells were left bathing in the dish with FSW for 5-10 minutes before the further treatment or imaging.

Sperm staining with Hoechst 33342

After the dry sperm was diluted in seawater, the final concentration of 5 μ M Hoechst 33342 (Sigma) was used to stain the sperm solution. The sperm was incubated in the presence of the DNA dye for 1 minute at 17 °C before it was used to inseminate the

eggs. The number of the egg-incorporated sperm was counted in epifluorescence microscopy, by applying the UV light (330 nm).

Treatments and microinjection of starfish oocytes and eggs with various agents

Ionomycin (calcium ionophore, Invitrogen) was diluted in DMSO (stock solution of 5mM) and was used in the final concentration of 5 μ M. The ionophore was first diluted to the final concentration and then added to the experiment-chamber in which oocytes or eggs were kept in a small amount of seawater. When microinjected into the oocytes, the ionomycin was diluted in the injection buffer and the concentration used was 500 μ M and 50 μ M. The control eggs were microinjected with 5 μ M InsP₃ (Molecular Probes), or with the injection buffer only.

The calcium ionophore A23187 (Sigma Aldrich) was prepared in DMSO and added to the eggs by bath incubation in seawater at a final concentration of 40 μ M for 20 minutes.

LAT-A was purchased from Invitrogen, and dissolved in DMSO (stock solution 3mM). Starfish eggs were incubated in the seawater containing 3 μ M LAT-A for 20 minutes and after the treatment the drug was removed by washing the eggs with a fresh seawater.

Nicotine (Sigma Aldrich) was suspended in the distilled water, and added at a final concentration of 300 μ M to the seawater containing oocytes that had been exposed to the maturation hormone 1-methyladenine for 40 minutes. After 40 minutes of incubation of the oocytes in seawater containing nicotine the drug was washed out and then mature eggs were further analyzed. Control oocytes were kept in the 1-methyladenine for 40 minutes and then transferred to fresh seawater, and after 40 minutes the experiment was performed.

Cold phalloidin (Invitrogen) was diluted in methanol, and was microinjected into the eggs at a final pipette concentration of 3 mM. The eggs were incubated for 15 minutes before doing the experiment. Control eggs were microinjected with the DMSO.

Staining of fertilized eggs with the lipophilic dye FM 1-43

Starfish (*A. pectinifera*) and sea urchin (*P. lividus*) eggs were treated with 1M citric acid for 30 sec to remove the jelly coat and then were washed with FSW. The eggs were then stained by incubating with 4 or 1 mM of FM 1-43 (Molecular Probes), respectively, in FSW for 2 minutes. After rinse with FSW, the eggs were imaged with confocal microscopy for real-time monitoring.

Preparation and microinjection of the proteins

Some of the proteins used in this study were expressed and purified from *E. coli* in collaboration with Dr. Chun at the Stazione Zoologica Anton Dohrn. The proteins obtained in our laboratory following dialysis in the injection buffer were: the RFP-PH, R40A-RFP, GFP-Utr and RFP. Anti-depactin antibody was generously donated by Dr. Issei Mabuchi and was diluted in the injection buffer before microinjection. Human gelsolin protein was purchased from Cytoskeleton, Inc, and was suspended in nanopure water. The concentrations of each protein used are indicated in the text and in the figures.

Data processing

The quantified Ca^{2+} signal at a given time point was normalized to the baseline fluorescence (F_0) following the formula $F_{\text{rel}} = [F - F_0] / F_0$, where F represents the average fluorescence level of the entire oocyte and F_0 is the reference fluorescence detected in the last image of a Ca^{2+} measurement before UV irradiation. In case when two dyes were used to measure the Ca^{2+} increase (Clacium Green and Rhodamine Red) the relative fluorescence was calculated using the formula $F_{\text{rel}} = [F_{\text{REL}(\text{green})} - F_{\text{REL}(\text{red})}] / F_{\text{RATIO}}$, where the recorded relative fluorescence for Red, $F_{\text{REL}(\text{red})} = F - F_0 / F_0$ was subtracted from relative fluorescence for Green, $F_{\text{REL}(\text{green})} = F - F_0 / F_0$ and then normalized against the ratio of the reference images for Green and Red acquired before the first detected Ca^{2+} signal, $F_{\text{RATIO}} = F_0(\text{green}) / F_0(\text{red})$. Fluorescence of Ca^{2+} images were analyzed with the MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA).

The numerical MetaMorph data were compiled and analyzed with Excel of Microsoft Office 2003. The average and variation of the data were reported as mean \pm standard deviation in all cases. The paired t-test and the one-way ANOVA were performed by use of Prism 3.0 (GraphPad Software, La Jolla, CA, USA), and the P-values smaller than 0.05 ($P < 0.05$) were considered statistically significant.

CHAPTER III

The role of the actin-binding proteins and PIP₂ in regulation of the intracellular Ca²⁺ signaling and egg activation in starfish

Results

Alteration in the plasma membrane PIP₂ level affects the cortical granules translocation during meiotic maturation, Ca²⁺-signaling and vitelline envelope elevation at fertilization

This part of the thesis addresses the question of how the fine alteration of the actin cytoskeleton structure by PIP₂ and actin-binding proteins can affect the intracellular Ca²⁺ signaling and egg activation at fertilization. PIP₂ is enriched in the plasma membrane, and is hydrolyzed by PLC to form InsP₃ and DAG. PIP₂ is also known to bind and modify the function of the actin-modulating proteins such as cofilin (Yonezawa et al., 1990), Arp2/3 complex and the Wiskott-Aldrich syndrome protein (Higgs and Pollard, 1999), profilin (Janmey, 1998), as well as gelsolin (Sun et al., 1999). Therefore, by changing the level or the accessibility of the plasma membrane PIP₂, it is possible to influence the activity of the actin-binding proteins, and thus affect the actin cytoskeleton. To sequester the plasma membrane PIP₂, I have used RFP-tagged fusion protein containing the plekstrin homology (PH) domain of rat PLC- δ 1. This PH domain (1-140 aa) has been shown to bind with high affinity and specificity to PIP₂, both, *in vivo* (Stauffer et al., 1998; Varnai and Balla, 1998) and *in vitro* (Lemmon et al., 1995).

One of the remarkable morphological changes observed during meiotic maturation in starfish oocytes is the actin cytoskeleton rearrangement (Santella et al., 2008; Santella and Chun, 2011). In parallel, the cortical granules that have been translocated from the inner cytoplasm toward the plasma membrane during oocyte growth, by the end of the GVBD become attached underneath to the plasma membrane. Cortical granules positioning beneath the plasma membrane and the Ca²⁺-induced extrusion of their content

into the perivitelline space at fertilization were demonstrated to be dependent on the proper actin cytoskeleton organization (Santella et al., 1999; Puppo et al., 2008). In order to determine whether sequestration of the plasma membrane PIP₂ can affect the orientation of the cortical granules, immature oocytes were microinjected with RFP-PH (18 µg/µl) and induced to undergo meiotic maturation by addition of 1-MA (10 µM). As shown in the electron microscopy image (Fig. III 1b) when the starfish oocytes undergo meiotic maturation in the presence of the RFP-PH, the cortical granules fail to align perpendicularly to the plasma membrane by the end of the GVBD. In contrast, when the control protein with one site mutation at Arg 40 of the PH domain (R40A-RFP, 18 µg/µl), which does not bind to PIP₂, was microinjected into the GV stage oocyte, the normal ordering and positioning of the cortical granules took place beneath the plasma membrane after 1 hour of exposure to 1-MA (Fig. III 1a). Hence, PIP₂ is directly implicated in the cortical granules distribution during starfish oocytes meiotic maturation.

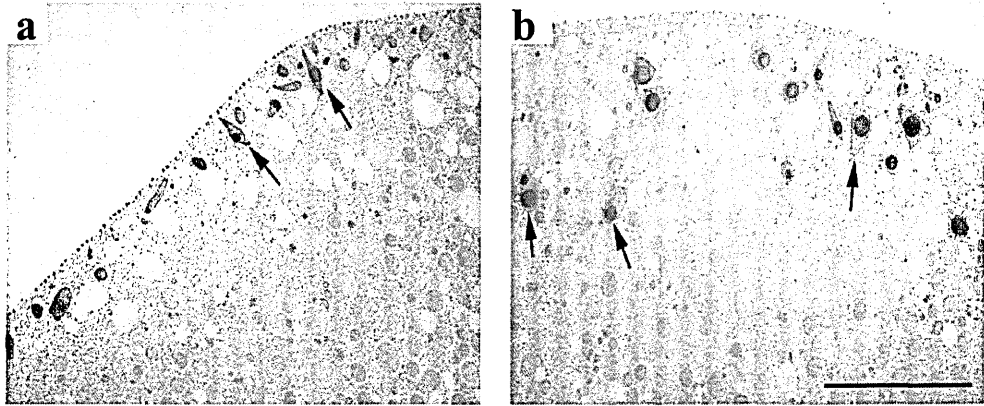


Figure III 1. Sequestration of PIP₂ affects cortical granules orientation during meiotic maturation. Transmission electron microscopic images show the ultrastructure of the *A. aurantiacus* eggs matured in the presence of R40A-RFP (18 µg/µl) or RFP-PH proteins (18 µg/µl). (a) In the egg microinjected with R40A-RFP at the GV stage, cortical granules (arrows) are intimately apposed to the plasma membrane after 1 hour of exposure to maturation hormone 1-MA. (b) In the eggs microinjected with RFP-PH, cortical granules failed to align perpendicularly to the plasma membrane by the end of the meiotic maturation. Scale bar, 10µm.

Because InsP_3 is produced rapidly in the fertilized eggs (Turner et al., 1984), it is considered to be the major Ca^{2+} -releasing second messenger in this physiological process. Plasma membrane PIP_2 is linked to InsP_3 in a several ways. PIP_2 serves as a substrate for PLC to produce InsP_3 during fertilization (Swann and Whitaker, 1986). On the other hand, in *in vitro* experiments was shown that PIP_2 rather suppresses InsP_3 -dependent Ca^{2+} signaling (Lupu et al., 1998). Furthermore, PIP_2 modulates the structure and the activities of the cortical actin cytoskeleton in the starfish eggs (Chun et al., 2010) and may also affect the Ca^{2+} signaling at fertilization. In order to elucidate these seemingly contradictory roles of PIP_2 in intracellular Ca^{2+} signaling, I have studied how PIP_2 contributes to the optimization of the intracellular Ca^{2+} releasing mechanism during meiotic maturation. To this end, immature oocytes were co-injected with RFP-PH (or control R40A-RFP) and the mixture of calcium dye and Rhodamine Red and stimulated with 1-MA (10 μM) for 1 hour. Then mature eggs were inseminated to monitor any changes in the pattern of the sperm-induced Ca^{2+} signaling. As shown (Fig. III 2a), the amplitude of the sperm-induced intracellular Ca^{2+} increase in the eggs that have undergone meiotic maturation in the presence of RFP-PH was significantly higher (1.08 ± 0.07 RFU, $n=6$) compared to the control eggs preinjected with the mutant protein R40A-RFP (0.77 ± 0.14 RFU, $n=5$, $P<0.05$). However, the kinetics of the Ca^{2+} increase was not affected, since the average length of time required for reaching the Ca^{2+} peak was not significantly different in the experimental (108 ± 44 sec, $n=6$) and control eggs (93.2 ± 48.9 sec, $n=5$, $P=0.5$). In line with the data that PIP_2 affects the cortical granules orientation during the meiotic maturation (Fig. III 1b), the eggs in which the RFP-PH was injected failed the normal fertilization envelope formation (Fig. III 2c). As expected, the transmission light image (Fig. III 2c) showed that all of the eggs preinjected with the RFP-PH displayed partial elevation of the vitelline layer (100%, $n=6$), demonstrating either small membrane elevation on the entire egg surface or a partial elevation only on one side of the egg (Fig.

III 2c). On the other hand, only one out of five eggs (25 %) preinjected with R40A-RFP displayed impaired elevation of the vitelline envelope (Fig. III 2c).

Alternatively, when the RFP-PH was used at lower concentrations as a probe to only visualize, but not sequester PIP₂ in starfish eggs, an increase of PIP₂ was found to take place in the plasma membrane in the sperm-activated eggs (Chun et al., 2010). The increased level of PIP₂ in the plasma membrane was shown to be linked with the formation of numerous actin-positive spikes traversing the perivitelline layer of the fertilized egg (Chun et al., 2010). To examine whether the spikes formation is limited only to a certain species or a detection method, I have applied an alternative method. Mature starfish eggs of *A. pectinifera* and eggs from sea urchin *P. lividus* were briefly stained with a lipophilic dye that selectively delineates the plasma membrane prior to fertilization. As shown in figure III 3, spikes formation in the perivitelline layer after 3 minutes of insemination were visualized by FM 1-43 both in fertilized starfish (Fig. III 3a and 3b) and sea urchin eggs (Fig. III 3c and 3d). In the same time scale starfish eggs were activated more slowly than sea urchin eggs, but the staining pattern of the perivitelline space was similar.

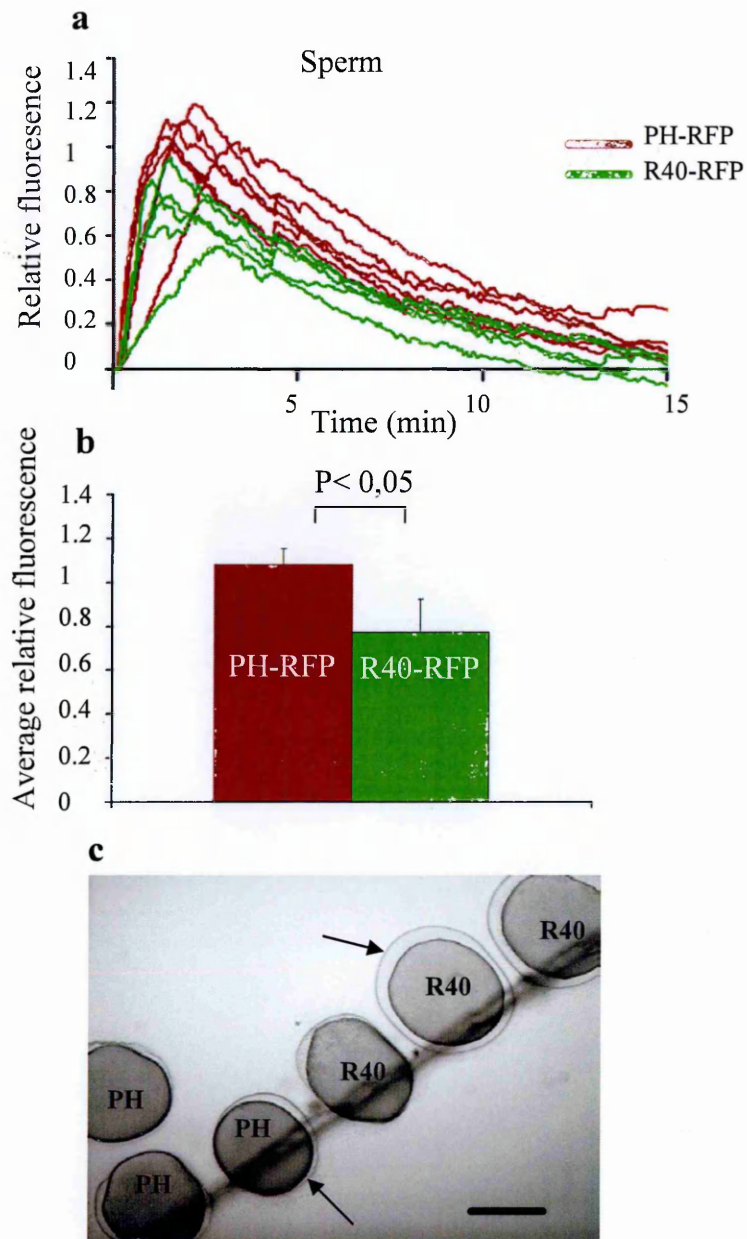


Figure III 2. Sequestration of plasma membrane PIP_2 leads to an increased intracellular Ca^{2+} response at fertilization, but with a partial elevation of the vitelline layer. *A. aurantiacus* oocytes were loaded with a Calcium Green / Rhodamine Red, and microinjected either with R40A-RFP (18 $\mu\text{g}/\mu\text{l}$) or RFP-PH (18 $\mu\text{g}/\mu\text{l}$). The oocytes were then exposed to 1-MA for 1 hour, and then fertilized. **(a)** Relative fluorescent Ca^{2+} signals were quantified in the eggs injected with R40A-RFP (green curves) and RFP-PH (brown curves). **(b)** The magnitude of the Ca^{2+} response in the RFP-PH (1.08 ± 0.07 RFU, $n=6$) microinjected eggs was significantly higher ($P<0.05$) than that of R40A-RFP (0.77 ± 0.14 RFU, $n=5$). **(c)** Bright field view of the eggs 20 minutes after fertilization shows the partial and full vitelline layer elevation in experimental and control eggs. Scale bar, 100 μm .

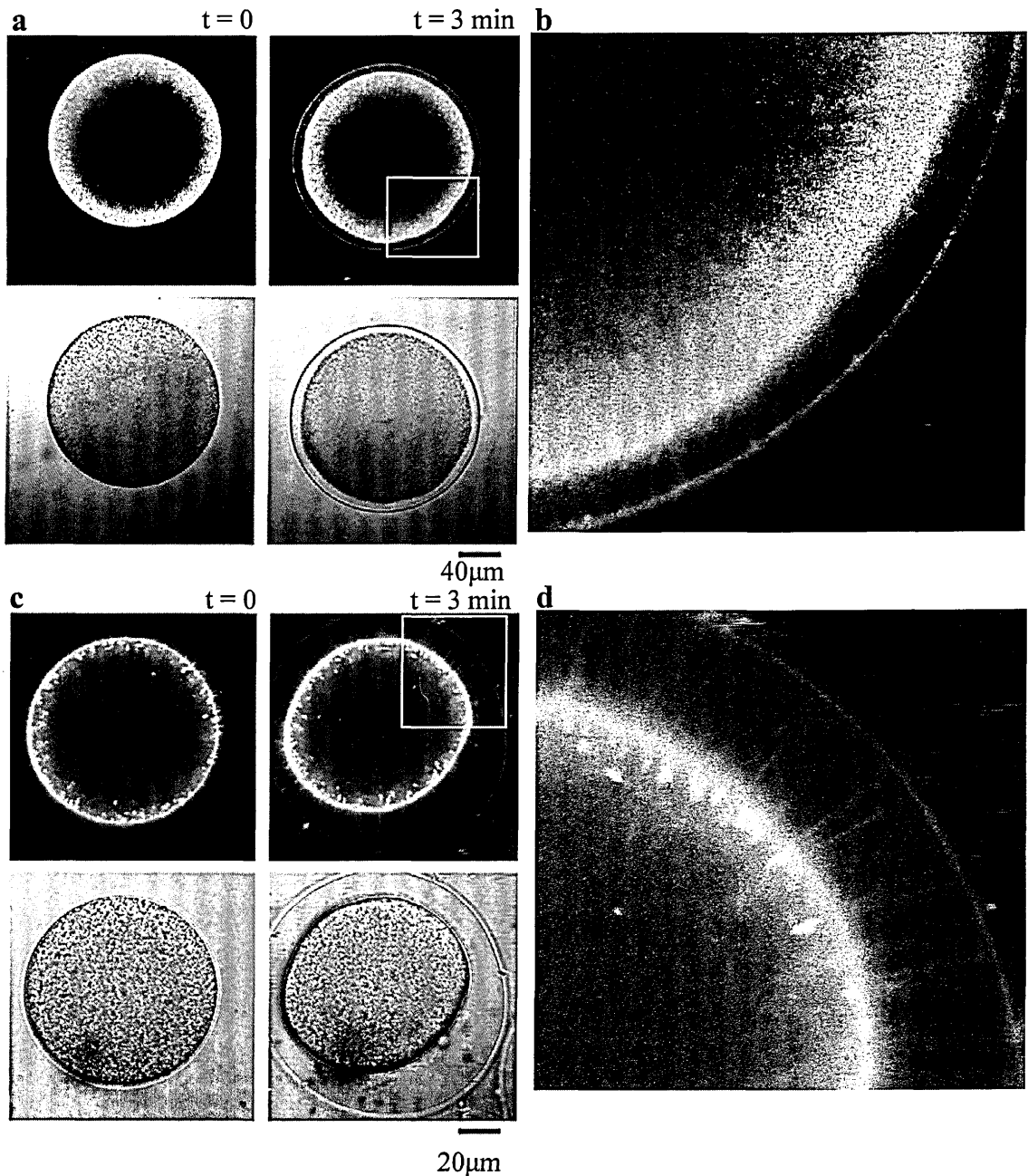


Figure III 3. Spikes formation in the perivitelline layer is not limited to starfish eggs but is also present in fertilized sea urchin eggs. (a) Starfish (*A. pectinifera*) eggs were pretreated with 4 mM lipophilic dye FM 1-43, then washed with natural seawater and imaged with confocal microscopy 3 minutes before and after of the addition of the sperm. Scale bar: 40 μm . **(b)** The mafnified view of the squared area in panel **a**. **(c)** Sea urchin (*P. lividus*) eggs pretreated with 1 mM FM 1-43, then washed with natural seawater and imaged with confocal microscopy 3 minutes before and after of the addition of the sperm. Scale bar: 20 μm . **(d)** The mafnified view of the squared area in panel **c**.

Sequestration of PIP₂ prevents eggs activation mediated by actin depolymerizing drug Latrunculin-A

Exposure of mature *Astropecten auranciacus* eggs to the actin depolymerizing drug LAT-A leads to a massive intracellular Ca²⁺ increase which is followed by a cortical granules exocytosis and vitelline layer elevation (Lim et al., 2002). This intracellular Ca²⁺ mobilization by LAT-A was suggested to be mediated by the InsP₃R since their antagonist heparin inhibits the Ca²⁺ increase evoked by LAT-A (Lim et al., 2002). However, in a recent report (Puppo et al., 2008) it was clearly shown that, heparin, besides its effect on the InsP₃R, can also alter the structure of the actin cytoskeleton in the subplasmalemmal domain of the egg. Thus, the inhibition of the LAT-A-induced Ca²⁺ response by heparin may be attributed not only to its pharmacological effect on the InsP₃R but also to its affect on actin cytoskeleton. PIP₂ has been known to regulate the function of the proteins involved in exocytosis (Schiavo et al., 1996; Chung et al., 1998; Grishanin et al., 2002) and in various cell types (Eberhard et.al., 1990; Hey et al., 1995). In order to see how PIP₂ is implicated in the process of the cortical granules exocytosis in starfish eggs, I have studied its role in the eggs activated by actin depolymerizing agent LAT-A.

As the presence of the RFP-PH in mature starfish eggs has been already demonstrated to alter the structure of the actin cytoskeleton at the egg cortex (Chun et al., 2010), I aimed to examine whether these changes can influence the LAT-A-stimulated egg activation. Therefore, mature eggs were microinjected with RFP-PH (18 µg/µl) and incubated for 15 minutes before being exposed to the actin depolymerizing drug LAT-A (3µM) for 20 minutes (Fig. III 4). In two independent experiments performed, only 15.3% (n=26) of the eggs that had been preinjected with the RFP-PH protein underwent activation (cortical granules exocytosis), whereas all of the eggs preinjected with the control RFP (18µg/µl) protein become activated (100%, n=29). Since the experimental eggs were microinjected with RFP-PH after GVBD when the cortical granules translocation and attachment beneath the plasma membrane had occurred normally, the failure of LAT-A to

activate these eggs was not due to the lack of the cortical granules beneath the plasma membrane. As shown on the electron microscopy image (Fig. III 5b), even though incubated for 20 minutes in the seawater containing 3 μ M LAT-A, the mature eggs microinjected with RFP-PH demonstrated failed egg activation with the cortical granules intact and tightly apposed to the plasma membrane. On the other hand, as judged by the electron-dense material of the cortical granules present in the perivitelline space of activated egg (Fig. III 5a), the microinjection of the control RFP protein into the mature eggs did not interfere with the LAT-A to exert its action and induce cortical granules exocytosis. Thus, masking the plasma membrane PIP₂ by the PH-domain interferes with the LAT-A ability to cause cortical granule exocytosis and vitelline layer elevation in mature starfish eggs.

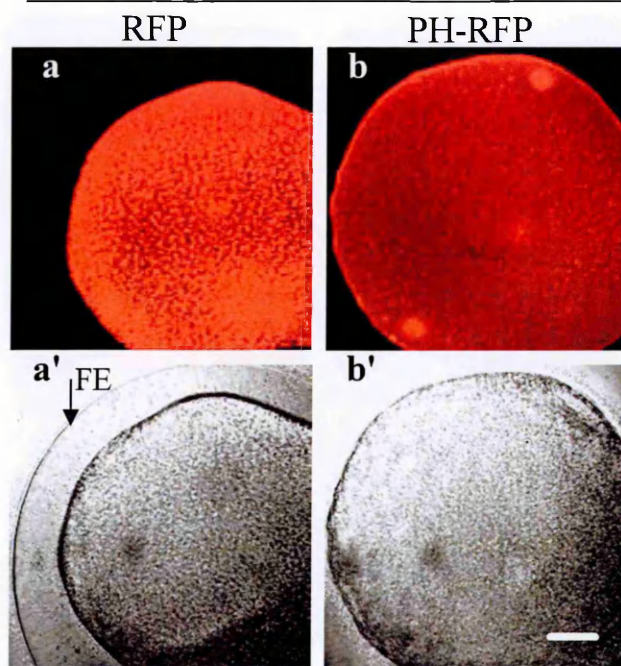


Figure III 4. Sequestration of the plasma membrane PIP_2 blocks the egg activation by LAT-A. Mature *A. auranciacus* eggs were microinjected either with RFP-PH ($18 \mu\text{g}/\mu\text{l}$) or with RFP ($18 \mu\text{g}/\mu\text{l}$) prior to treatment with LAT-A ($3 \mu\text{M}$). **(a)** Confocal microscopic images of RFP fluorescence and **(a')** the corresponding bright field view of the activated egg 20 minutes after the exposure to LAT-A (FE-fertilization envelope). **(b)** Confocal microscopic image taken 20 minutes after LAT-A treatment of mature egg microinjected with PH-RFP protein. **(b')** Bright view of the same egg from **b**, demonstrating the failure of LAT-A to induce vitelline layer elevation. Scale bar, $20\mu\text{m}$.

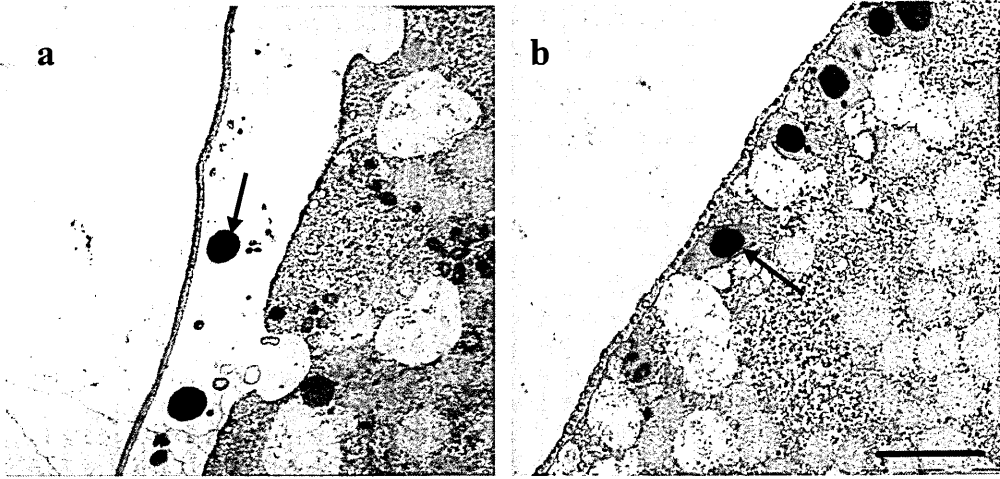


Figure III 5. Sequestration of plasma membrane PIP_2 blocks the cortical granules exocytosis induced by the treatment with LAT-A. The ultrastructural images of the cortex of mature eggs after 20 minutes of exposure to LAT-A. Mature *A. aurantiacus* eggs were microinjected either with RFP (18 $\mu\text{g}/\mu\text{l}$), or, with RFP-PH (18 $\mu\text{g}/\mu\text{l}$), exposed to LAT-A (3 μM) for 20 minutes, washed and then fixed with 1-gluteraldehyde and analyzed with the electron microscopy. **(a)** LAT-A treatment caused cortical granules (arrows) exocytosis in mature egg preinjected with the control RFP protein. **(b)** LAT-A did not cause any structural changes in the cortex of the RFP-PH preinjected mature eggs. Scale bar, 10 μm .

The effect of the actin-binding protein depactin on the regulation of the intracellular Ca^{2+} signaling and the meiotic maturation

Precise remodeling of the actin cytoskeleton is regulated by the combinatorial action of many actin-binding proteins. Besides the indirect way of modifying the actin cytoskeleton networks by altering the availability of the plasma membrane PIP_2 that interacts and modulates a host of actin-binding proteins, I also employed a more targeted approach using a specific function-blocking antibody against depactin, the actin-depolymerizing protein present in *A. aranciacus* oocytes (Nusco et al., 2006) to monitor the effect of the blockade of this actin-modulating protein on intracellular Ca^{2+} increase at fertilization.

Ca^{2+} release from the InsP_3 -sensitive stores is enhanced during meiotic maturation of starfish oocytes (Lim et al., 2003), and the increased sensitivity correlates with the dynamic rearrangement of F-actin (Lim et al., 2003), suggesting that the actin cytoskeleton distribution may be linked to the enhanced Ca^{2+} response. Recent data from our laboratory have demonstrated that the alterations of the actin cytoskeleton by introducing an actin-binding protein cofilin into the starfish oocytes could augment the InsP_3 -stimulated intracellular Ca^{2+} increase (Nusco et al., 2006). Since InsP_3 is considered to be the major second messenger responsible for the Ca^{2+} release at fertilization, I have studied whether the actin cytoskeleton reorganization induced by the actin-binding protein could affect the sensitization of the InsP_3 -mediated Ca^{2+} signaling during meiotic maturation.

The anti-depactin antibody (a generous gift from Prof. Issei Mabuchi) is a specific function-blocking agent for depactin, a starfish version of cofilin. It has been generated against starfish (*Asterias amurensis*) depactin, and was shown to detect a single band of 17 kDa in the western blot analysis of *A. auranciacus* (Nusco et al., 2006). This antibody is supposed to interfere with the ability of the endogenous depactin to interact with its physiological partners, and consequently to alter F-actin dynamics. Therefore, to test whether the antibody influences InsP_3 -mediated Ca^{2+} signals during meiotic maturation I

have microinjected the antibody (4 $\mu\text{g}/\mu\text{l}$) into GV stage (immature) oocytes that have been pre-loaded with caged InsP_3 (5 μM) and calcium dye, and performed a photoactivation of the caged InsP_3 in the oocytes before and after stimulation with the maturation hormone 1-MA. Firstly, it was noticed that the oocytes microinjected with the anti-depactin antibody displayed a time delay in the GVBD. If the approximate time at which the control oocytes preinjected with the immunoglobulin-G (IgG) (4 $\mu\text{g}/\mu\text{l}$) underwent GVBD was 65 minutes ($n=32$) (Fig. III 6, pink curve), for the oocytes preinjected with the antibody was 72 minutes ($n=37$) (Fig. III 6, green curve). The approximate time needed for the GVBD to occur in the intact oocytes was 62 minutes ($n=30$) (Fig. III 6, blue curve) indicating that the time delay was due to the action of the antibody. In addition, despite the delay of GVBD in the examined oocytes preinjected with the depactin antibody, was noticed that the nucleoplasm did not intermix well with the cytoplasm (not shown).

Since the alterations of the actin cytoskeleton induced by the depactin antibody have already been shown both in immature (GV stage) and mature eggs (Puppo, 2009), I have continued to investigate whether the changes in the immature oocytes could somehow influence the InsP_3 -mediated Ca^{2+} release during maturation. Therefore, the control (microinjected with IgG) and experimental oocytes (microinjected with anti-depactin antibody) were co-injected with the mixture of a calcium dye (Calcium Green, 500 μM , pipette concentration) and caged InsP_3 (5 μM , pipette concentration) and exposed to UV light (330 nm) for 15 seconds at the three time points of the meiotic cycle: *i*) at the GV stage (prophase I) before addition of 1-MA; *ii*) after 40 minutes of 1-MA addition; and, *iii*) after 70 minutes after the exposure to 1-MA, when the oocytes had already undergone GVBD (metaphase I). In line with the sensitization of the InsP_3 -induced Ca^{2+} release during meiotic maturation, in the presence of the control IgG, the Ca^{2+} rise have been gradually increasing showing 0.38 ± 0.28 RFU ($n=8$) in GV stage oocytes, and 0.53 ± 0.41 RFU ($n=8$) in oocytes exposed to 1-MA for 40 minutes and 1.49 ± 0.09 RFU ($n=8$), in mature eggs (70 min after 1-MA) (Fig. III 7a and 8a). In parallel, the oocytes preinjected

with the depactin antibody showed slightly lower Ca^{2+} increase in all three time points compared to the control oocytes, but, the difference was statistically significant only in the mature eggs. The InsP_3 -induced Ca^{2+} increase in the GV stage oocytes microinjected with depactin antibody was 0.3 ± 0.18 RFU ($n=8$), whereas in oocytes exposed for 40 minutes to 1-MA 0.49 ± 0.12 RFU ($n=8$). When the InsP_3 was uncaged in mature eggs, 70 minutes after addition of 1-MA, the Ca^{2+} increase (1.31 ± 0.1 RFU, $n=8$) was statistically lower compared to the InsP_3 uncaging in the control eggs (1.49 ± 0.09 RFU, $n=8$, $P<0,05$). Interestingly, the time necessary for the Ca^{2+} increase to reach the maximum peak during the InsP_3 uncaging at the each time point of the maturation process, was always faster in the oocytes preinjected with the depactin antibody than in the control oocytes (Fig. III 8b). Moreover, as the amplitude of the InsP_3 -induced intracellular Ca^{2+} release during the meiotic maturation increases (Fig. III 8a), the time for reaching the maximum peak of the Ca^{2+} rise is being reduced both in control and experimental cells, respectively (Fig. III 8b). Hence, the fine actin cytoskeleton rearrangement caused by blocking the function of actin-binding protein depactin, induces changes in the kinetics and in the amplitude of the InsP_3 promoted Ca^{2+} release.

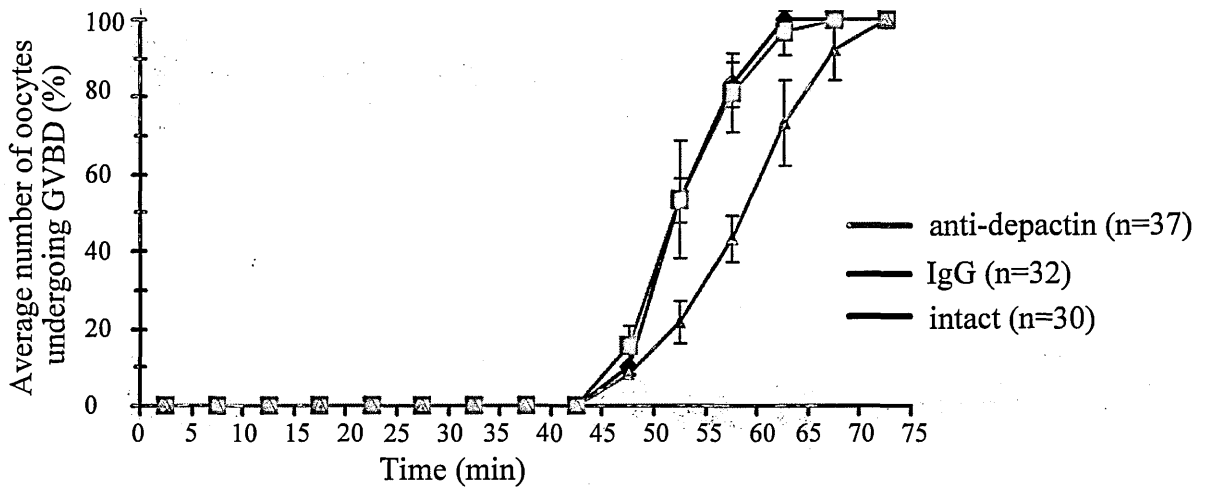


Figure III 6. Time course of the GVBD during meiotic maturation in the presence of anti-depactin antibody. The GV stage *A. aranciacus* (immature) oocytes were microinjected either with the control immunoglobulin-G (IgG) (4 $\mu\text{g}/\mu\text{l}$) protein (n=32) or with the anti-depactin antibody (4 $\mu\text{g}/\mu\text{l}$) (n=37). After 15 minutes of incubation, microinjected oocytes along with the intact oocytes (n=30) were stimulated by 1-MA to undergo meiotic maturation. For the oocytes injected with the anti-depactin antibody the approximate time at which examined oocytes underwent GVBD was ≈ 72 minutes (green curve), whereas for the control (IgG) and control intact oocytes was ≈ 67 minutes (pink curve) and ≈ 63 minutes (blue curve), respectively.

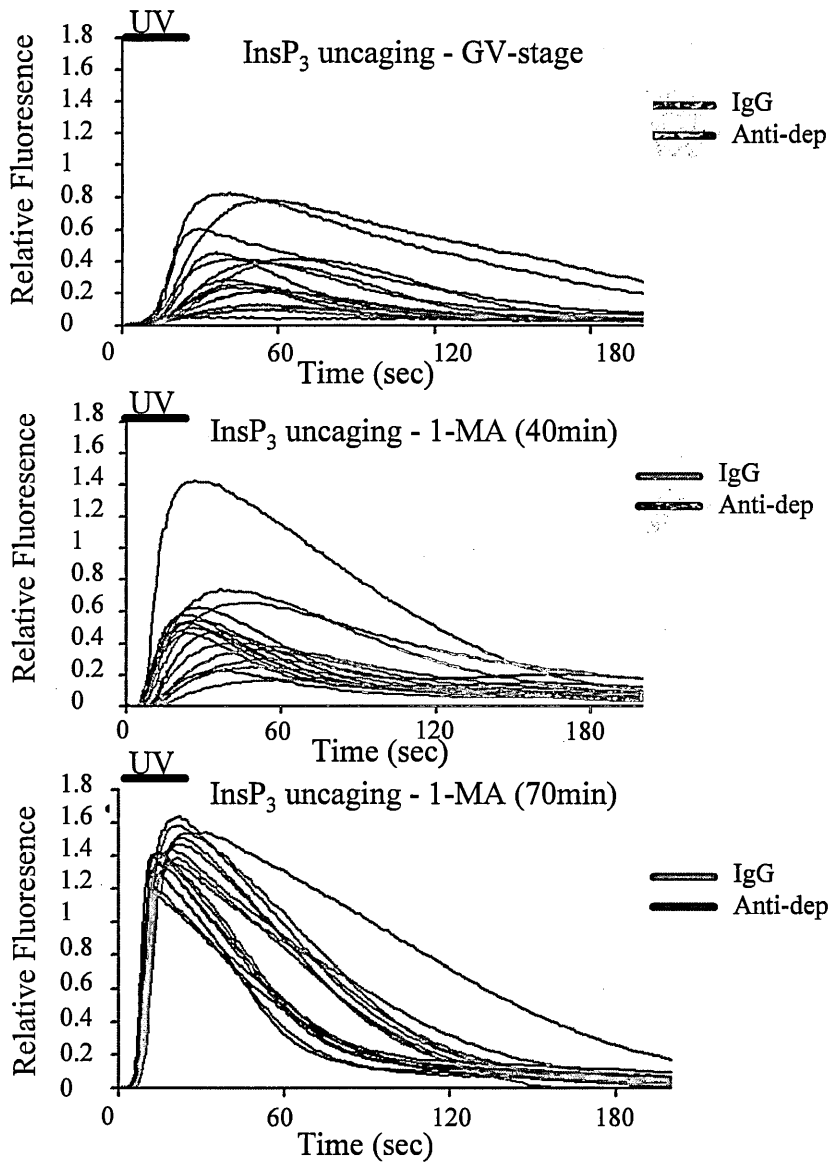


Figure III 7. Anti-depactin antibody decreases the InsP_3 -induced Ca^{2+} response in mature eggs. GV stage oocytes of the *A. aurantiacus* species were injected with a mixture of a Calcium Green (500 μM , pipette concentration) and caged InsP_3 (5 μM , pipette concentration). Then, 15 minutes before the addition of the 1-MA, one group of oocytes were injected with 4 $\mu\text{g}/\mu\text{l}$ immunoglobulin-G as a negative control and another group with 4 $\mu\text{g}/\mu\text{l}$ anti-depactin antibody. (a) The graphs show the kinetics of the relative fluorescent Ca^{2+} curves after InsP_3 -uncaging by exposure to UV light (330 nm) for 15 seconds performed on the CCD camera. Intracellular Ca^{2+} increase was measured in the GV stage oocytes, in the oocytes exposed to 1-MA for 40 and 70 minutes.

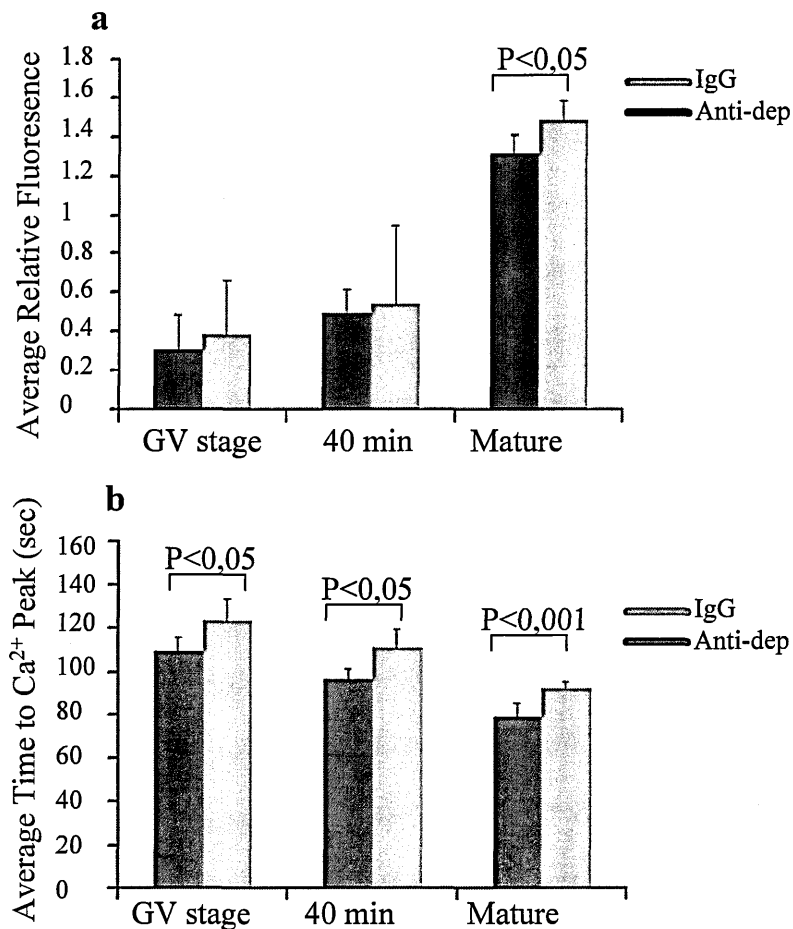


Figure III 8. Anti-depactin antibody accelerates the time of the Ca²⁺ rise during meiotic maturation and reduces the amplitude of the InsP₃-induced Ca²⁺ signaling in mature eggs. The experimental conditions are the same as in experiments shown in Figure III 6. **(a)** The bar histograms compare the average Ca²⁺ peaks for control (green bar) and experimental (brown bars) oocytes obtained during the photoactivation of caged InsP₃ in the GV stage oocytes, and in the oocytes exposed to 1-MA for 40 and 70 minutes. **(b)** The average time to reach the maximum Ca²⁺ peak in response to InsP₃ uncaging in the two groups of oocytes at three different time points of the cell cycle. The presence of the antibody at the three time points of the cell cycle showed statistically faster Ca²⁺ rise compare to the one in the control oocytes (P<0,05 and P<0,001).

Fertilization of starfish eggs in the presence of actin-modulating protein gelsolin

As it was demonstrated, blocking the function of depactin interferes with the InsP_3 - and sperm-induced intracellular Ca^{2+} increase. In order to extend the knowledge and examine if another actin-binding protein may have an impact on the intracellular Ca^{2+} signaling in starfish eggs, I have inseminated mature eggs microinjected with gelsolin. Gelsolin is a 80 kDa Ca^{2+} - and PIP_2 -regulated actin-binding protein that cuts actin filaments and caps the severed end, leading to Ca^{2+} -dependent remodelling of the actin cytoskeleton (Sun et al., 1999). A human recombinant gelsolin (1 mg/ml, pipette concentration) was microinjected into mature starfish eggs, and the eggs were inseminated after 20 minutes incubation. The relative fluorescent measurements of the Ca^{2+} dye in the fertilized eggs showed the cortical flash which represents a massive Ca^{2+} increase beneath the plasma membrane induced by the sperm at the initial moments of the sperm-egg interaction was affected (Fig. III 9b). The presence of gelsolin in the fertilized eggs reduced to 42 % (3 out of 7) the occurrence of the cortical flash compare to 70 % (7 out of 10) occurrence in the control eggs (Fig. III 9b). However, the presence of gelsolin affected neither the amplitude nor the kinetics of the Ca^{2+} rise. The height of the intracellular Ca^{2+} increase in experimental eggs (1.28 ± 0.12 RFU, $n=12$) was not significantly different to that in the control eggs (1.33 ± 0.09 , $n=15$, $P=0.26$) microinjected with the denaturated gelsolin (incubated at 95 °C for 5min) (Fig. III 9a and c). In parallel, the kinetics of the Ca^{2+} increase was not affected, as there was no difference in the time for the Ca^{2+} rise to reach the peak in control (100.6 ± 21.4 sec, $n=15$) and experimental eggs (112.6 ± 27.6 sec, $n=12$, $P=0.23$). Despite the comparably high intracellular Ca^{2+} increase induced by the fertilizing sperm, the gelsolin preinjection inhibited the formation of the fertilization envelope. Only 33 % (4 eggs out of 12) of the eggs microinjected with gelsolin underwent normal fertilization envelope formation compared with the 100 % (15 eggs out of 15) in the control (denaturated gelsolin) eggs (Fig. III 9d). Hence, the actin-modulating protein

gelsolin affected the cortical granules exocytosis and the cortical flash, while the amplitude and the kinetics of the sperm-induced intracellular Ca^{2+} seemed not to be much altered.

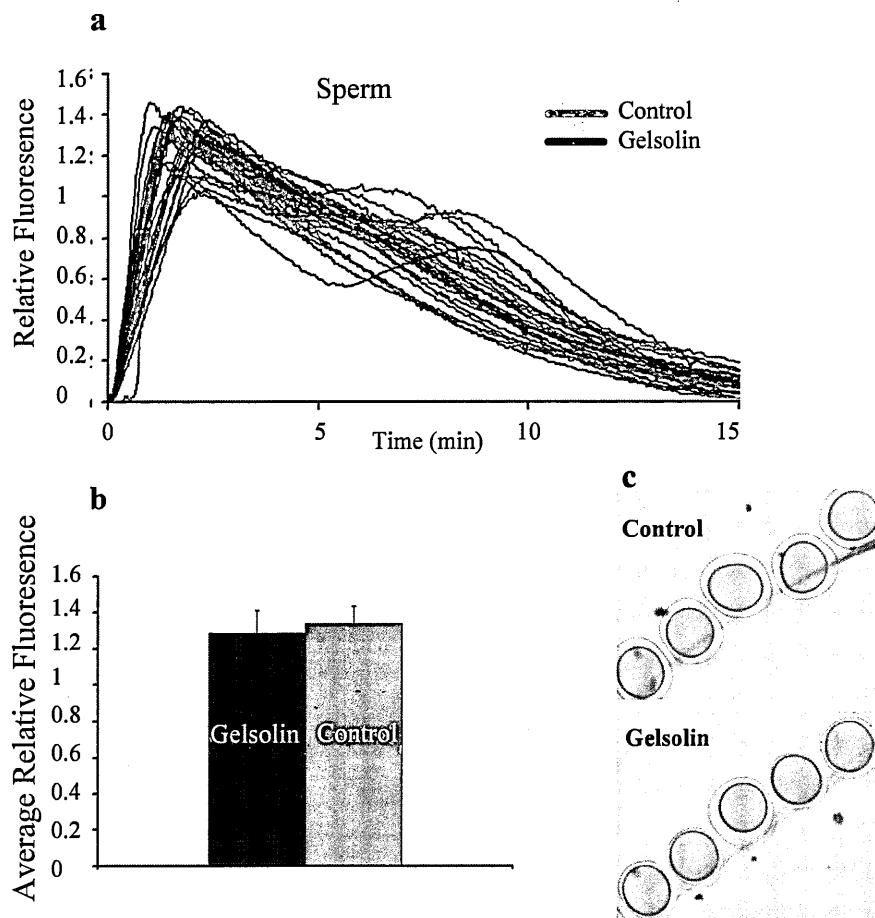


Figure III 9. Sperm-induced intracellular Ca^{2+} increase in the eggs microinjected with actin-severing protein-gelsolin. Mature *A. aurantiacus* eggs were microinjected with Calcium Green / Rhodamine Red and either with the denaturated (95 °C, 20 minutes) human gelsolin (1 mg/ml, pipette concentration), or, with the gelsolin (1 mg/ml, pipette concentration). Eggs were incubated for 20 minutes and then fertilized. (a) Relative fluorescence signals of the calcium dye detected under the CCD camera in fertilized control (15 green curves) or experimental (12 brown curves) mature eggs. (b) Relative fluorescence pseudo-colored images of cortical flash in control egg, and abolished cortical flash in the egg preinjected with gelsolin. (c) Averaged relative fluorescent signals of the amplitude of the Ca^{2+} -rise in control (1.33 ± 0.09 RFU, $n=15$) and experimental eggs (1.28 ± 0.12 RFU, $P=0.26$, $n=12$). (d) Transmission light images taken with the CCD camera, showing the partial (in the presence of gelsolin) and a normal vitelline layer elevation (in the presence of a denaturated gelsolin) in the eggs 20 minutes after fertilization. Scale bar, 100 μm .

Actin-binding protein utrophin, as a tool for the F-actin visualization in starfish eggs

Despite an important role of the actin-binding proteins in regulation of the polymerization/depolymerization dynamics of the actin cytoskeleton, the fluorescently tagged actin-binding proteins can be a useful molecular probe for detecting and visualizing the rate of actin cytoskeleton changes in different experimental models (Gerisch et al., 1995; Edwards et al., 1997; Kost et al., 1998). Therefore, I have further studied how the actin cytoskeleton changes during fertilization of starfish eggs can be visualized by specific F-actin binding protein.

I have used a probe developed on the basis of the F-actin-binding domain of the human protein-utrophin, with the aim to obtain another molecular tool for visualization of the actin cytoskeleton in a live oocyte. Utrophin is known as a non-muscle counterpart of dystrophin (Winder et al., 1995). Its calponin-homology domain (CH) was shown to bind to F-actin *in vitro* (Rybakova and Ervasti, 2005) and *in vivo* in various cell types (Burkel et al., 2007). By using a green fluorescent protein-tagged CH of utrophin (GFP-Utr) was demonstrated that it binds and visualizes the changes of F-actin during meiotic maturation in starfish and embryonic development in sea urchins with no apparent evidence to interfere with any of the above mentioned processes (Burkel et al., 2007).

The GFP-Utr was expressed and purified from *E. coli* (in collaboration with Dr. Chun) and was used for microinjections into the starfish oocytes. Microinjected immature oocytes and mature eggs of *A. aurantiacus* were observed under the confocal microscope to confirm the binding of the recombinant protein to the F-actin. As shown in the figure III 10a, immature oocytes with microinjected GFP-Utr (12 mg/ml, pipette concentration) showed a clear staining of F-actin around the germinal vesicle and in the cytoplasm as short microfilaments, while the staining in the plasma membrane often took patched form. On the other hand, mature eggs displayed a clear perpendicular staining of the microfilaments in the subplasmalemmal region and the short actin filaments in the inner cytoplasm (Fig. III 10b). In order to check whether this probe could visualize the active F-

actin remodelling in starfish oocytes, the immature oocytes preinjected with GFP-Utr were exposed to the actin-depolymerization agent LAT-A (3 μ M). After 20 minutes of incubation, oocytes were washed and observed under the confocal microscope. As shown on the confocal microscopic image (Fig. III 11b), a drastic actin depolymerization induced by the drug can be clearly visualized by the GFP-Utr around the nucleus and in the cortex of the immature oocytes. On the other hand, the time matching control oocyte not treated with LAT-A, showed a characteristic staining of the F-actin in the cortex and around the nucleus (Fig. III 11a).

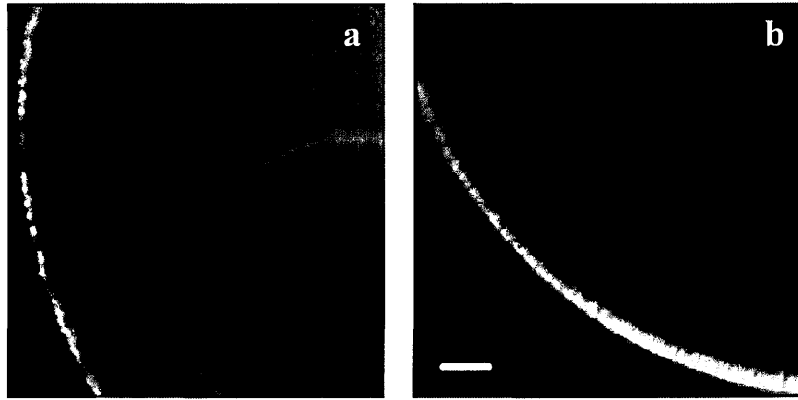


Figure III 10. F-actin staining in immature and mature starfish oocytes vizualized by the GFP-Utr. Immature *A. auranciacus* oocytes (a) and mature eggs (b) were microinjected with GFP-Utr (12 mg/ml) and observed under the confocal microscope 10 min later. (a) F-actin staining with the recombinant protein in the GV stage oocyte revealed predominant staining near the plasma membrane and nuclear envelope. Note that the staining in certain areas of the subplasmalemmal region takes a patchy form. (b) In mature egg, the staining showed the perpendicularly oriented F-actin fibers in the egg cortex. Scale bar, 10 μ m.

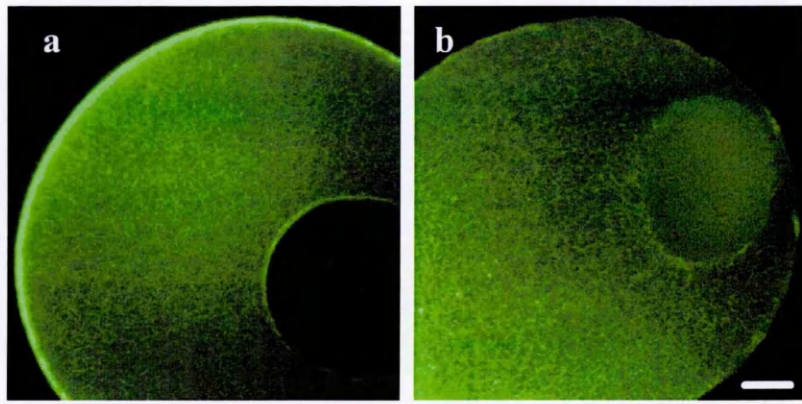


Figure III 11. GFP-Utr visualizes the actin depolymerization induced by LAT-A

Confocal microscopic images of the control (non-treated) and immature *A. auranciacus* oocytes treated with LAT-A. **(a)** Control immature oocyte microinjected with GFP-Utr (12 mg/ml) and observed with the confocal microscope after 25 minutes. **(b)** Immature oocyte microinjected with GFP-Utr (12 mg/ml), incubated for 5 minutes in the fresh seawater and then treated with 3 μ M LAT-A for 20 minutes. After the treatment the LAT-A was washed out, and the oocytes were observed with the confocal microscope. Scale bar, 20 μ m.

Now the next goal was to examine whether the probe could detect the dynamic changes of the actin cytoskeleton in a physiological process like fertilization. For this purpose, mature eggs were microinjected with GFP-Utr (12 mg/ml, pipette concentration) and after 5 minutes of incubation in seawater were inseminated. The real-time changes in the structure of the actin cytoskeleton of the fertilized eggs were monitored under the confocal microscope (Fig. III 12). At variance with the data of the F-actin staining with the fluorescent phalloidin (Chun et al., 2010; Vasilev et al., 2012), the GFP-Utr probe did not visualize the F-actin spikes formed in the perivitelline space, nor the cortical actin fibers that centripetally migrate in the egg cortex at the time of the sperm entry (Terasaki, 1996; Chun et al., 2010; Vasilev et al., 2012). However, the probe visualized the rich actin meshwork inside the fertilization cone on the surface of the fertilized egg 5 and 8 minutes after insemination (Fig. III 12b, c and f). In addition, when the fertilized eggs preinjected with GFP-Utr were observed at higher magnification, a staining around the cortical vesicles was observed (Fig. III 13b' and c') at the time of active exocytosis of cortical granules, i.e. vitelline layer elevation (Fig. III 13b and c). The staining of the cortical vesicles visualized by the probe was gradually decreasing and by 22 minutes after insemination only a few cortical vesicles were surrounded with F-actin (Fig. III 13d'). Taken together, these results suggest that GFP-Utr might selectively labels the actin filaments that are physically associated with the membrane such as plasma membrane, nuclear envelope and the cortical vesicles in motion.

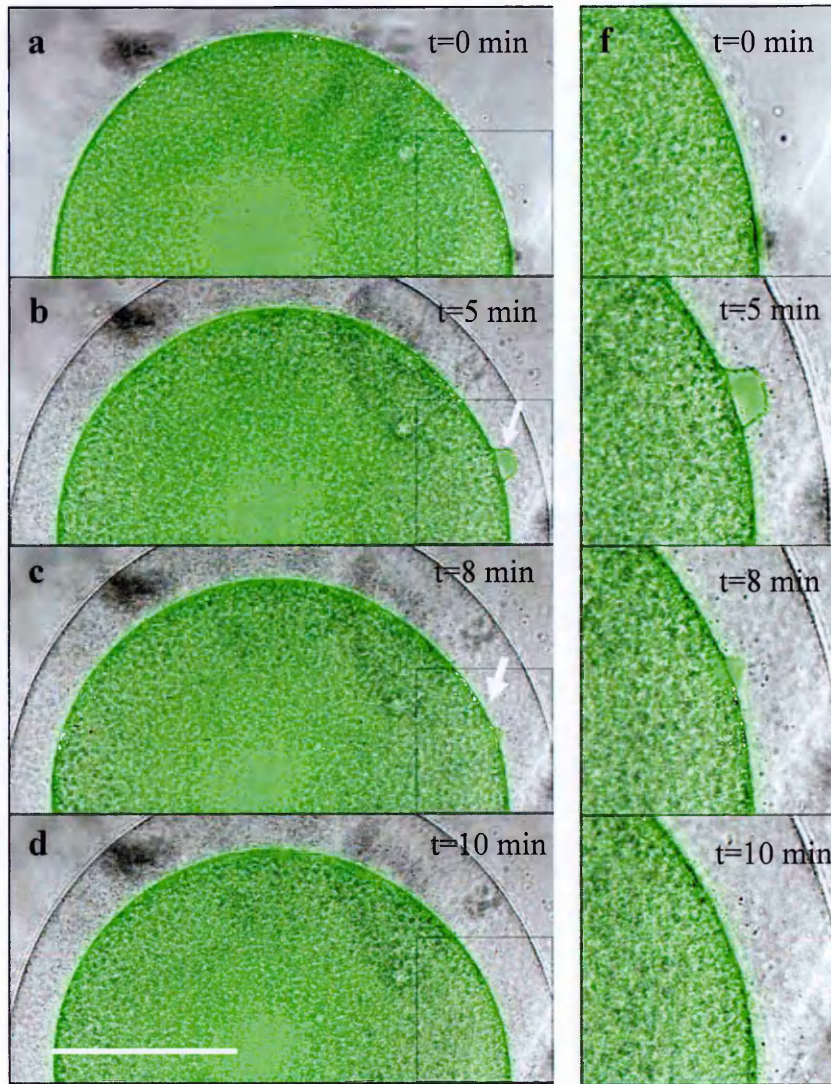


Figure III 12. F-actin visualization by GFP-Utr during fertilization of starfish eggs.

Merged images of the confocal and transmission light microscopic images. Mature eggs of *A. aurantiacus* were microinjected with GFP-Utr (12 mg/ml). After 5 minutes of incubation, the eggs were observed under the confocal microscope just before the sperm addition (a) and (b) 5, (c) 8 and (d) 10 minutes after sperm addition. Scale bar, 100 μ m. (f) The panel represents magnified views of the squared areas from the panels a, b, c and d.

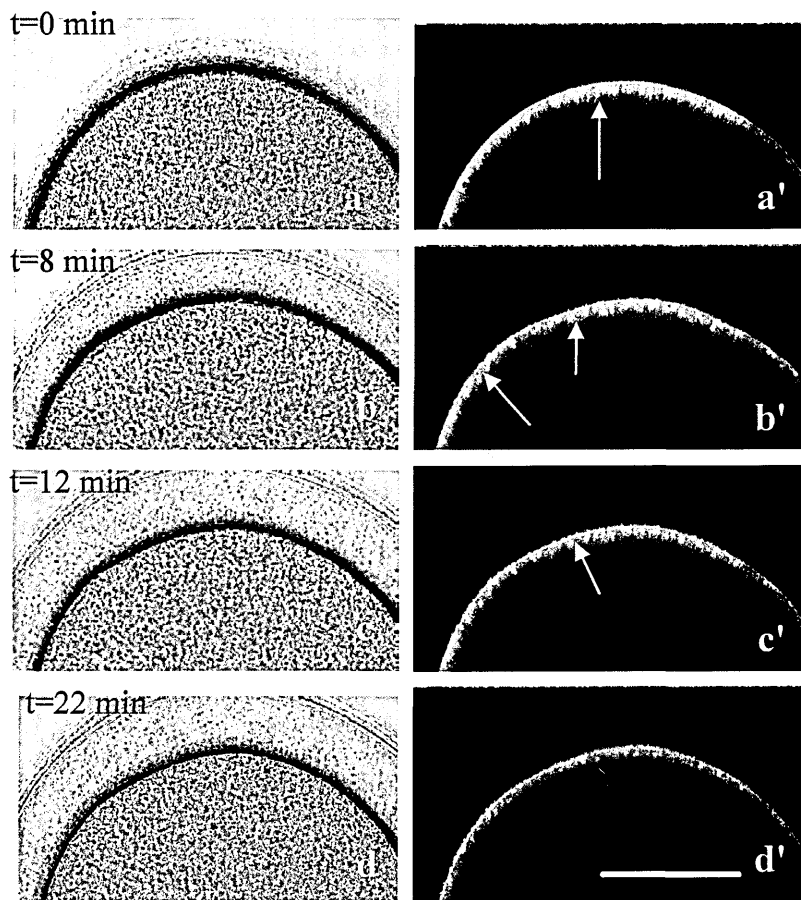


Figure III 13. GFP-Utr binds to F-actin that is formed around the cortical vesicles during fertilization. Mature eggs of *A. aranciacus* were microinjected with GFP-Utr (12 mg/ml, pipette concentration) and inseminated after 5 minutes of incubation. The bright field views of the egg (**a**, **b**, **c**, **d**) are shown together with the correspondent fluorescent confocal images of the same egg (**a'**, **b'**, **c'**, **d'**) before ($t=0$) and after the sperm addition. (**a'**) The probe revealed the perpendicularly oriented microfilaments in the cortex of unfertilized mature egg. After the egg becomes activated by the fertilizing sperm after (**b'**) 8 and (**c'**) 12 minutes the probe binds around the numerous vesicles in the cortex. (**d'**) After 22 minutes of the sperm addition, the number of the cortical vesicles visualized by the probe was dramatically reduced. Scale bar, 100 μm .

Discussion for Chapter III

The PH-RFP fusion protein binds to PIP₂, and thereby renders the PIP₂ inaccessible to other PIP₂-interacting proteins, which include a host of actin-binding proteins (Sechi and Wehland 2000; Yin and Janmey, 2003). PIP₂ sequestration during meiotic maturation of starfish oocytes appeared to inhibit the cortical granules orientation (Fig. III 1b). The positioning and distribution of the cortical granules towards the plasma membrane during meiotic maturation is dependent on the actin cytoskeleton (Santella et al., 1999; Wessel et al., 2002). Since PIP₂ is able to influence the function of many actin-binding proteins, it is conceivable that the intracellular perpendicular positioning beneath the plasma membrane was interfered with because of the PIP₂ sequestration by the PH-domain that may have dislodged certain class of actin-binding proteins.

Considering that PIP₂ is a substrate for the PLC to produce InsP₃ to support the Ca²⁺ signaling during fertilization, one would expect a lower Ca²⁺ signals by masking the plasma membrane PIP₂ and making it less available for hydrolysis to produce InsP₃. Indeed, when mature eggs were microinjected with the RFP-PH before fertilization, the sperm-induced Ca²⁺ signals showed significant delay in the kinetics and slightly lowered amplitude of the response than the control eggs preinjected with the R40A mutant protein (Chun et al., 2010). Hence, by lowering the level of the free PIP₂ at the plasma membrane with RFP-PH, the pattern of Ca²⁺ signaling at fertilization was subtly changed. Another interpretation of the data in Chun et al (2010) is that the subtle changes in the actin cytoskeleton caused by PIP₂ sequestration near the egg plasma membrane may have affected the intracellular Ca²⁺ signaling, as has been numerously demonstrated in the starfish oocytes (Lim et al., 2003; Nusco et al., 2006; Chun and Santella, 2009). Given that in starfish oocytes the actin cytoskeleton undergoes dramatic rearrangement during meiotic maturation, altering the function of the actin-binding proteins by PIP₂ sequestration may be responsible for the alteration in the intracellular Ca²⁺ release at fertilization. Moreover, as

the PIP₂ was reported to regulate the ion channels (Lupu et al., 1998; Suh et al., 2008) it is conceivable that its sequestration have interfered with ion channel activities.

In this thesis, I have used a slightly different experimental paradigm to test the effects of PIP₂ sequestration by RFP-PH that has been present in the starfish eggs during the maturation process. When oocytes microinjected with RFP-PH were induced to resume meiotic cycle with 1-MA and fertilized 1h later, the level of intracellular Ca²⁺ increase was surprisingly higher than in the control eggs at fertilization (Fig. III 2a and b). One way to interpret the data is that the intracellular Ca²⁺-releasing mechanism that is optimized during meiotic maturation is even more sensitized in the presence of RFP-PH. Alternatively, the enhanced Ca²⁺ response might be linked to the structural changes that were induced by RFP-PH. In this sense, it would be interesting to know if the distribution of InsP₃R on the ER was changed by the treatment, or if the actin cytoskeleton is also changed in its structure in the egg cortex.

Although eggs microinjected with the PH-domain at the GV stage displayed a higher Ca²⁺ increase at fertilization, the vitelline envelope elevation was impaired (Fig. III 2c). Because the vitelline layer elevation takes place as a result of the exocytosis of the cortical granules, this result was expected since the PH-domain interferes with the translocation of the cortical granules beneath the plasma membrane during meiotic maturation (Fig. III 1b). In addition, a successful elevation of the fertilization envelope may require extension of the actin spikes in the perivitelline space, which was suggested to provide a mechanical force for the process (Chun et al., 2010). Hence, by altering the PIP₂ level, PH-RFP might have altered the actin dynamics at the perivitelline space and thereby affected the elevation process of the fertilization envelope. It would be interesting to study whether the eggs that had been microinjected with PH-RFP at the GV stage oocytes would be polyspermic as was expected from the lack of full elevation of the fertilization envelope.

As it was mentioned before, incubation of mature starfish eggs with the LAT-A induces intracellular Ca^{2+} increase and cortical granules exocytosis that leads to the vitelline layer elevation (Lim et al., 2002). LAT-A stimulated activation of mature eggs is inhibited when the eggs are preinjected with the PH-domain (Fig. III 4b'). Consequently, the cortical granules remained attached beneath the plasma membrane even after prolonged incubation (20 minutes) in the sea water containing LAT-A (Fig. III 4b). It is known that PIP_2 regulates actin binding proteins that may interfere with the F-actin present in the cell cortex (Di Paolo and De Camilli, 2006). When actin is highly polymerized in the cortex of the starfish eggs either by heparin or JAS (Puppo et al., 2008), the subsequent exposure to LAT-A failed to induce the intracellular Ca^{2+} increase and activate the eggs (Lim et al., 2002; Lim et al., 2003). Hence, the failure of LAT-A to induce cortical granules discharge in the perivitelline space in the RFP-PH preinjected starfish eggs might be similarly due to the changes of the actin cytoskeleton.

Depactin is a 17 kDa protein that has been identified in starfish (Mabuchi, 1981) and specifically binds to actin molecules (Sato and Mabuchi, 1989). Depactin detaches the actin monomers from the polymer of both ends of the filament, and forms 1 to 1 actin-depactin complex (Mabuchi, 1983) thus inducing actin cytoskeleton depolymerization *in vitro* (Takagi et al., 1988). On the other hand, depactin can also accelerate the rate of polymerization of actin probably by producing many short filaments that can serve as a nuclei or roots for a new filaments formation (Mabuchi, 1983).

Here, in this chapter I reported on the significant delay of the GVBD in the oocytes microinjected with depactin antibody (Fig. III 6). By the end of the GVBD was observed an incomplete mixing of the nucleoplasm with the cytoplasm. Previously, our laboratory has demonstrated that the microinjection of the depactin-antibody into the GV stage oocytes induced formation of actin fibers formation inside the germinal vesicle (nucleus) (Puppo, 2009).

While nuclear actin does not seem to form microfilaments in normal conditions, it has been suggested that actin in the nucleus might play various functions such as participation in the transcription machinery, nucleocytoplasmic transport, structural support and local translocation of the chromatin (Bettinger et al., 2004; Lenart et al., 2005). Actin may play an important role in the nuclear assembly, as it was shown in a cell-free assay by using *Xenopus laevis* egg extracts (Krauss et al., 2002). The role in establishing and/or maintaining the nuclear structure was suggested to be through interactions with the nuclear lamina proteins (Sasseville and Langelier, 1998; Lattanzi et al., 2003; Bettinger et al., 2004). Moreover, in the context of our findings that cofilin translocates itself inside the nucleus (Ohta et al., 1989; Pendleton et al., 2003), the functionally homologous depactin might do the same and exert its effect in the nucleus. Hence, in the presence of the function-blocking antibody, depactin was expected to be inhibited and might have thereby derepressed the formation of the actin fibers in the nucleus. Hence, the shift of actin assembly and disassembly dynamics in the nucleus of the starfish eggs by the depactin antibody could explain the failed intermixing of the cytoplasmic and nuclear environments.

Starfish eggs which had undergone meiotic maturation in the presence of the function-blocking antibody against the depactin showed significant decrease in the amplitude of the InsP_3 -induced intracellular Ca^{2+} release (Fig. III 7a and III 8a). Not only the amplitude of the Ca^{2+} rise, but also the kinetics of the Ca^{2+} increase was affected, being much faster compared to control eggs (Fig. III 8b). These results are in line with the blocking effect of the antibody of the function of the endogenous depactin (cofilin), as the published data demonstrate that the addition of the cofilin (homologous protein to depactin) into the cytoplasm of starfish oocytes induces significantly higher amplitude of the InsP_3 -induced Ca^{2+} release (Nusco et al., 2006). There may be several ways by which depactin could affect the intracellular Ca^{2+} signaling in response to InsP_3 . Depactin could modulate the activity of the InsP_3R indirectly by remodelling the cytoskeleton and microenvironment. In line with this possibility, it has been demonstrated that the Ca^{2+}

influx across the plasma membrane is regulated by cofilin (Rueckschloss et al., 2001; Redondo et al., 2006). Since the increased sensitivity of the InsP₃R in starfish eggs is not likely to be due to the increased charge of the Ca²⁺ stores during the meiotic process (Chiba et al., 1990; Lim et al., 2003), nor to overexpression or redistribution of the InsP₃R (Iwasaki et al., 2002), the fine regulation of the actin cytoskeleton dynamics might be implicated in the sensitization of the InsP₃-mediated Ca²⁺ release. Indeed, the filamentous actin was shown to link to the InsP₃R and thereby influence the Ca²⁺ signaling elicited by InsP₃ (Turvey et al., 2004). Another possibility is that depactin has affected the Ca²⁺ liberation from the actin fibers, as proposed by the F-actin-Ca²⁺ store hypothesis (Lange and Brandt, 1996). Finally, it would be interesting to investigate whether depactin could directly bind to and fine-tune the activity of the InsP₃R.

Another actin-modulating protein used in this chapter to rearrange the actin cytoskeleton was gelsolin. It is a 95 kDa cytosolic protein that severs actin filaments when it is activated by Ca²⁺, and after cleaving the actin filaments, gelsolin remains tightly bound to the barbed ends (Yin et al., 1981b; Cooper et al., 1987; Janmey and Stossel, 1987). Microinjection of gelsolin into mature eggs affected neither the amplitude of the Ca²⁺ rise, nor the kinetics of the intracellular Ca²⁺ release at fertilization (Fig. III 9a and c). However, the initial Ca²⁺ increase beneath the plasma membrane, the so called ‘cortical flash’ was affected along with the normal fertilization envelope formation (Fig. III 9b and d). Given that the cortical flash at fertilization is a result of the Ca²⁺ influx (Moccia et al., 2003; Churchill et al., 2003), gelsolin-induced actin cytoskeleton remodelling could affect the calcium channels on the plasma membrane. In support of the idea, it was reported that in the neuronal cells lacking gelsolin, the magnitude of the Ca²⁺ influx is affected upon stimulation (Furukawa et al., 1997). On the other hand, a detrimental effect on the vitelline layer elevation may be explained by the depolymerizing effect that gelsolin might have had on the actin assembly in the egg cortex. As the vitelline layer elevation is accompanied by the extensive actin cytoskeleton polymerization to sustain the elongation of the spikes in

the perivitelline space of the fertilization envelope, the gelsolin-induced changes in the actin cytoskeleton may have affected this process.

The GFP-Utr can be used as a molecular marker for visualization of the F-actin in starfish oocytes and eggs. This F-actin staining probe very similarly reflects the state of the actin cytoskeleton in GV stage oocytes and in mature eggs, as was previously shown with the staining of F-actin with the Alexa-conjugated phalloidin (Kyojuka et al., 2008; Puppo et al., 2008). The probe binds to the F-actin during a dynamic remodelling of the actin cytoskeleton induced either with the actin-modifying agent (e.g. LAT-A, Fig. III 11), or during fertilization of starfish eggs (Fig. III 12 and 13). The novelty observed by this probe was the data showing that GFP-Utr actively binds around the vesicles-like ring structures in the egg cortex at fertilization (Fig. III 13). As the visualization of these structures with the probe was diminishing with the time after the egg activation, the probe supposedly marked the F-actin that was generated around the cortical granules presumably to facilitate their exocytosis. In line with this data is the F-actin staining visible around the fused white vesicles and cortical granules in the ionomycin pretreated starfish eggs (Vasilev et al., 2012). However, the probe did not mark the cortical actin migration and the spikes formation in the perivitelline space of the fertilization envelope. It will be interesting to determine if the probe may have some effect on the Ca^{2+} signaling and for the further development of the *A. aranciatus* zygotes.

CHAPTER IV

Effects of ionomycin on egg activation and early development in starfish

Results

Ionomycin induces intracellular Ca^{2+} increase in starfish oocytes and eggs by release from internal stores and from Ca^{2+} influx

The fusion of the egg and sperm plasma membranes gives rise to a several physiological changes in the fertilized eggs, e.g. rapid changes in the actin cytoskeleton structure, alteration of the electrical property of the plasma membrane, cortical granules exocytosis, initiation of DNA replication and protein synthesis (Trimmer et al.1986; Epel, 1990). Practically in all animal species, these cytological and biochemical changes, termed 'egg activation', are accompanied by a significant increase in intracellular Ca^{2+} that propagates in the eggs as a single wave or oscillating waves (Ridgway et al., 1977; Miyazaki, 1991; Jaffe, 2002; Santella et al., 2004). The original experiments performed in sea urchin eggs leading to the conclusion that the intracellular Ca^{2+} increase is the initial stimulus elicited by the sperm to trigger the egg activation were done by using the calcium ionophore A23187. Based on the ability of the A23187 to cause the cortical granules exocytosis and fertilization envelope formation in sea urchin eggs in the Ca^{2+} -free seawater (CaFSW) lead to the prevailing view that the intracellular Ca^{2+} plays the key role in initiating the cytological changes in fertilized eggs (Steinhardt and Epel, 1974). Nevertheless, Ca^{2+} -independent way might also exist and contribute to the egg activation at fertilization (Ciapa and Arnoult, 2011).

Ionomycin is a Ca^{2+} -selective ionophore that has been isolated from the bacterium *Streptomyces globatus* (Liu et al., 1978). It binds Ca^{2+} in one-to-one stoichiometry and support mostly electrically neutral change of Ca^{2+} for 2H^+ or other divalent cations such as Mg^{2+} , and thereby transfers Ca^{2+} ions across the vesicle membranes or through the water-lipid interface (Liu et al., 1978; Erdahl et al., 1994). Ionomycin is shown to be more specific and potent than A23187 (Kauffman et al., 1980),

but the precise mechanism of how it raises the Ca^{2+} level inside the cells is not clearly understood and still remains controversial (Morgan and Jacob, 1994). Ionomycin may exert its action on plasma membrane and induce Ca^{2+} influx, or act on the intracellular organelles to release Ca^{2+} . On the other hand, it may use an indirect way to potentiate the existing Ca^{2+} -mobilizing mechanisms or to activate an enzyme such as PLC that in turn cleaves PIP_2 to produce the Ca^{2+} -releasing second messenger InsP_3 . The action of ionomycin may depend on the concentration used, since it has ionophoretic effects at $>1\mu\text{M}$ (Mason and Grinstein, 1993; Morgan and Jacob, 1994).

Immature *A. aranciacus* starfish oocytes were microinjected with Calcium Green and Rhodamine Red mounted on the coverslip and monitored with the CCD camera for the intracellular Ca^{2+} changes. Immediately after the addition of ionomycin to the artificial sea water containing 10 mM Ca^{2+} (ASW), the intracellular calcium levels started to rise. The calcium signals were significantly increased in the cortical area just beneath the plasma membrane before spreading to the center of the oocyte (Fig. IV 1a). Eight out of nine oocytes displayed sharp rise and fall of Ca^{2+} in the entire cortical region (cortical flashes) 10.5 ± 2.9 seconds after the ionomycin addition (Fig. IV 1a, arrow), and by 5 minutes of exposure the Ca^{2+} signals arrived at the plateau (Fig. IV 1b). However, in the CaFSW the initial Ca^{2+} rise was significantly delayed (16.2 ± 5.3 sec, $n=11$) compared with oocytes treated in ASW (6.7 ± 2.9 sec, $n=9$, $P<0.0001$) (Fig. IV 1c). In agreement with the idea that the short-lasting (<2 sec) cortical flash represents a Ca^{2+} influx (Muallem et al., 1995; Lim et al., 2001; Moccia et al., 2003; Churchill et al., 2003), no cortical flashes were observed in the oocytes incubated with the same concentration of ionomycin ($5\mu\text{M}$) in the CaFSW (0 out of 11) (Fig. IV 1a). Furthermore, the amplitude of the Ca^{2+} peak in these oocytes was significantly lower (0.46 ± 0.12 FRU, $n=11$) than that of Ca^{2+} -containing sea water (0.86 ± 0.04 RFU, $n=9$, $P<0.0001$). In addition, upon reaching the peak in oocytes treated in Ca^{2+} -containing sea water, the Ca^{2+} increase was sustained on the high levels, compared to that of the oocytes incubated in CaFSW where the Ca^{2+} levels

fell back to the basal level within 15 minutes of exposure (Fig. IV 1b). Therefore, ionomycin-induced intracellular Ca^{2+} increase is a result of a release from the intracellular stores and a Ca^{2+} influx being responsible for maintaining the Ca^{2+} levels high after reaching the peak.

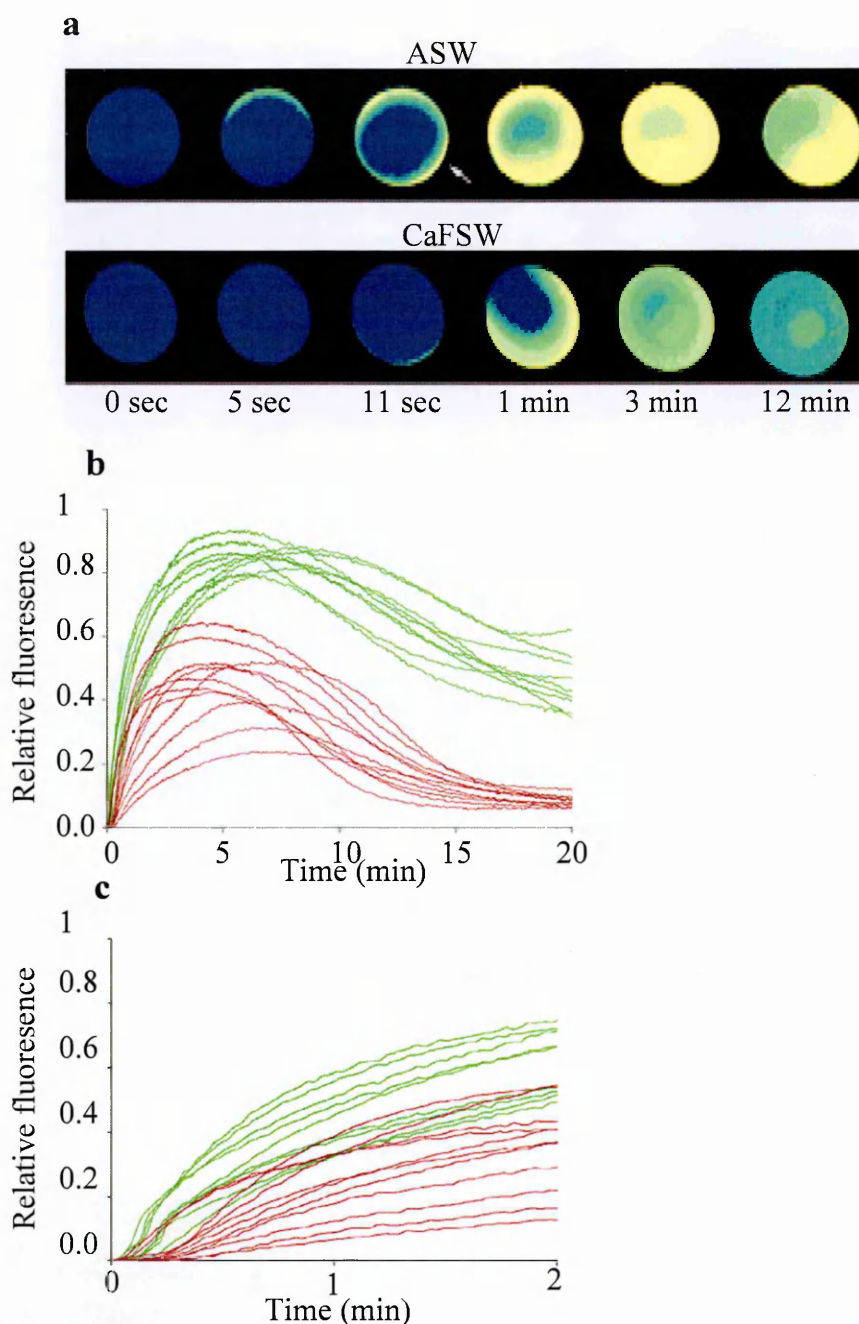


Figure IV 1. Changes of intracellular Ca^{2+} levels in the starfish oocytes exposed to ionomycin. *A. aranciatus* oocytes at the GV stage were microinjected with Calcium Green / Rhodamine Red and exposed to 5 μM ionomycin in ASW or in CaFSW. (a) The pseudocolored images of Ca^{2+} changes within the representative oocytes at several key time points. Indicated by an arrow is the cortical flash. (b). The dynamics of the released Ca^{2+} after addition of ionomycin in ASW and CaFSW are represented in green and brown curves, respectively. The moment of the first detectable Ca^{2+} signal was set to $t = 0$ in panels b and c. (c) The initial response of the oocytes to ionomycin in ASW (green curves) and CaFSW (brown curves).

Ionomycin treatment of immature oocytes causes retraction of microvilli and fusion of cortical vesicles

When treated with ionomycin, the GV stage starfish oocytes responded with a slight elevation of the vitelline layer (Fig. IV 2), as was previously shown in the context of ectoplasmic maturation for the oocytes exposed to a sub-threshold concentration of maturation hormone (Hirohashi et al., 2008; Terasaki and Runft, 2012). The most evident vesicles residing in the ectoplasm of the GV stage oocytes along with the migrating cortical granules are the so called ‘white vesicles’. It is not much known about their function, except the fact that their content may be needed for the fertilization envelope formation, as they are exocytosed during vitelline layer elevation at fertilization (Vasilev et al., 2012). In the oocytes that have been briefly exposed to 1-5 μM ionomycin, the white vesicles in the subplasmalemmal region have reduced in number and enlarged in size (Fig. IV 2a and b). When measuring the longer axis, the average diameter of the ‘white vesicles’ in the control oocytes was 1.2 μm whereas in the treated ones ranged from 4 to 8 μm . The TEM (transmission electron microscopy) image has clearly shown two intermediately large vesicles ($>4 \mu\text{m}$) forming a ‘peanut shell-shaped’ twin structure in the ionomycin-pretrated oocytes (Fig. IV 2c, blue arrows, right panel), supporting the idea that the formation of the bigger white vesicles is due to intervesicular fusion. Interestingly, close to the plasma membrane the white vesicles appeared to have engulfed the electron-dense materials, supposedly derived from the cortical granules (Fig. IV 2c, red arrows, right panel). Furthermore, the microvilli representing finger-like protrusions filled with actin filaments on the surface of the control oocytes (Fig. IV 2c, blue arrows, left panel) were completely absent in the ionomycin-treated oocytes, leaving behind a smooth plasma membrane (Fig. IV 2c, right panel). Finally, ionomycin treatment seems to selectively target the white vesicles and cortical granules because the other vesicles such as yolk platelets, which appear as most numerous and intermediately dark granules in the TEM (Fig. IV 2c), did not undergo much structural change.

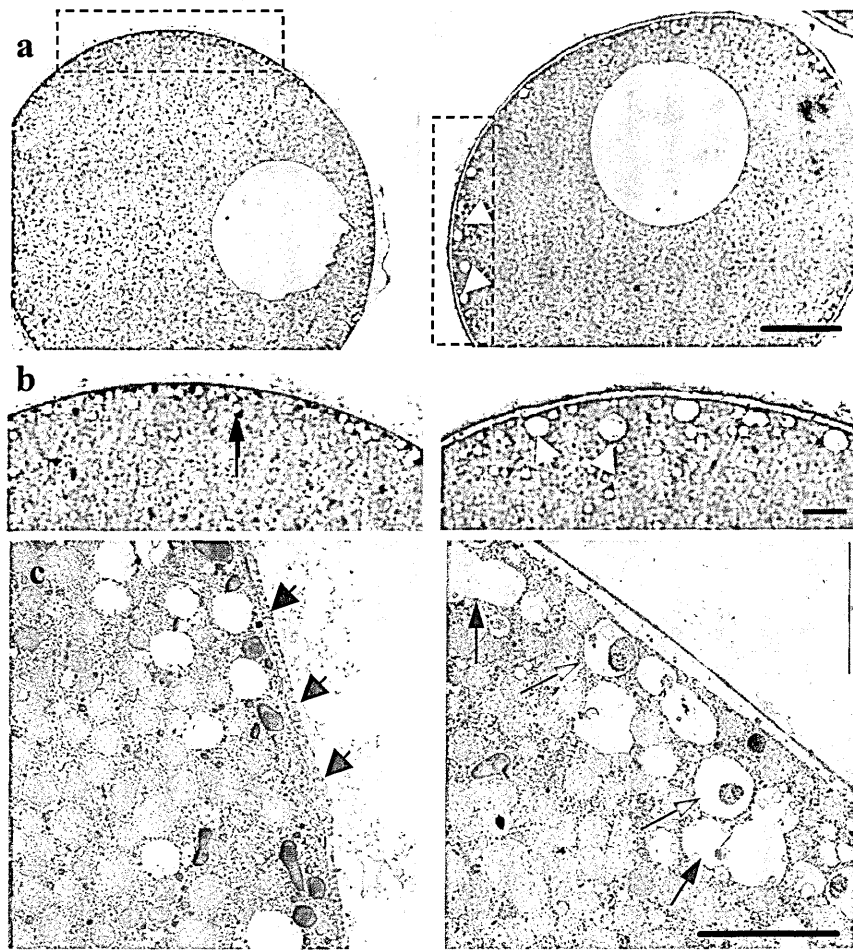


Figure IV 2. Morphological changes in the cortex of the starfish oocytes exposed to ionomycin. *A. aranciacus* oocytes at the GV stage were incubated for 5 minutes in natural seawater containing 5 μ M ionomycin and then fixed in 1 %-glutaraldehyde. (a) Bright field view in the light microscope of control oocyte (left panel) and treated oocyte (right panel). Scale bar, 50 μ m. (b) The magnified views of the dot-lined rectangular areas in panel a. The same large vesicles present in the treated oocyte in panel a were marked with yellow arrowheads (right panel). Cortical granules that appear as dark vesicles sized about 1 μ m (black arrow) in control oocyte (left panel) had largely disappeared in the oocytes briefly exposed to ionomycin (right panel). Scale bar, 10 μ m. (c) TEM image of the same batch of oocytes incubated in the natural sea water (left) or in the presence of 5 μ M ionomycin for 5 minutes (right). In the cross-section of the control oocytes (left) blue arrows indicate microvilli. In pretreated oocytes (right panel), red arrows indicate the white vesicles engulfing electron-dense cortical granules whereas blue arrows show the white vesicles at fusion. Scale bar, 10 μ m.

Immature oocytes and mature eggs of starfish undergo drastic rearrangement of the actin cytoskeleton upon ionomycin treatment

The disruption of the microvilli on the surface of the ionomycin-treated oocytes suggested that the alteration in the actin filaments took place. To examine that, I have microinjected Alexa Fluor 488-conjugated phalloidin into immature oocytes and monitored under the confocal microscope the changes in the actin cytoskeleton during the treatment. After 5 minutes of exposure to 5 μM ionomycin the actin cytoskeleton revealed dramatic rearrangement (Fig. IV 3a), whereas the F-actin structures of untreated oocytes remained virtually unchanged within the same timeframe (not shown). Hence, during the ionomycin-induced intracellular Ca^{2+} rise, the actin cytoskeleton underwent drastic rearrangement. As depicted in the figure IV 3a, in the inner cytoplasm, the actin cytoskeleton was highly polymerized, forming longer and thicker microfilament bundles and patches. At the same time, the typical actin filaments in the subplasmalemmal region that are intimately associated with plasma membrane (Fig. IV 3a, white arrow at $t=0$) disappeared by 5 minutes of exposure, indicating active depolymerization of the actin filaments in this subcellular domain. This structural reorganization of the actin cytoskeleton was maintained even after 20 minutes of exposure to the ionophore (data not shown). Hence, ionomycin remodels the actin cytoskeleton in the inner cytoplasm and in the cortical area in a different manner: accelerated polymerization in the inner cytoplasm and simultaneous fast and extensive depolymerization in the subplasmalemmal region. However, when GV stage oocytes were pretreated with ionomycin (5 minutes), washed and then let to resume the meiotic maturation for one hour in the presence of 1-MA (10 μM), the actin cytoskeleton structure was, to some extent, restored to the state of the control mature eggs (Fig. IV 3b). Nevertheless, the F-actin fibers typically observed perpendicular to the plasma membrane in the control mature eggs (Fig. IV 3b, arrowheads), were largely absent despite the restoration of the dense actin network in the region. Interestingly, phalloidin staining showed F-actin fibers cluster around the large vesicles formed by ionomycin (Fig. IV 3b,

arrows). Except for these structural changes in the ectoplasm, ionomycin pretreated oocytes underwent apparently normal meiotic maturation in the nucleus, with the GV breakdown occurring in the same time schedule (1 h) after 1-MA addition.

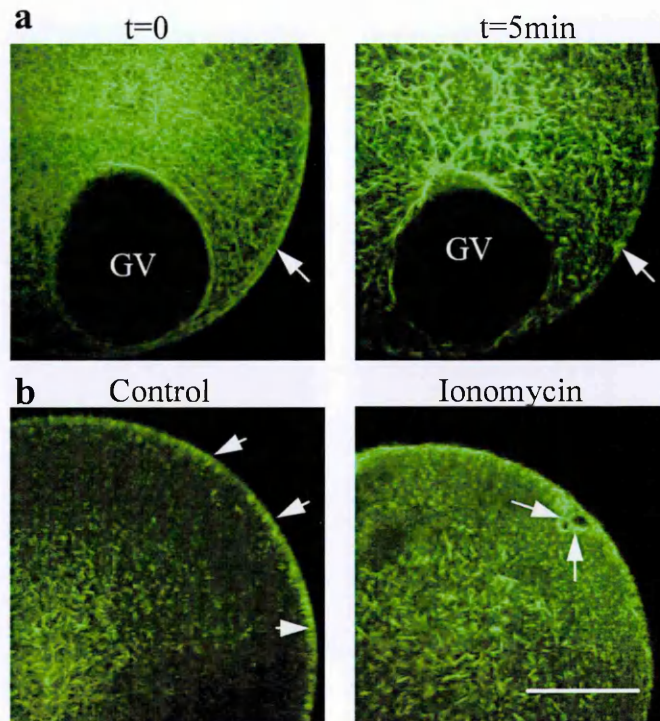


Figure IV 3. Ionomycin induces rapid rearrangement of the actin cytoskeleton. (a) *A. aranciacus* oocytes were microinjected with Alexa Fluor 488-conjugated phalloidin, exposed to 5 μ M ionomycin and monitored under the confocal microscope. The continuous layer of the subplasmalemmal actin network delineating the plasma membrane (arrow, $t=0$, left panel) had mostly disappeared in the same oocyte within 5 minutes after the treatment with ionomycin (right panel). In parallel, the actin filaments in the inner cytoplasm formed bundles and became much thicker and longer. (b) After 5 minutes of exposure to 5 μ M ionomycin, the oocytes were washed and induced to undergo meiotic maturation for 1 hour in the seawater containing 1-MA (10 μ M) and then observed with the confocal microscope. Arrowheads indicate the actin fibers in the subplasmalemmal region (left panel) and arrows indicate on the actin filaments around the big fused white vesicles (right panel). Scale bar, 50 μ m.

Short exposure of starfish oocytes to ionomycin causes disruption and exocytosis of cortical granules

As it was aforementioned, the brief exposure (3-5 minutes) to 5 μ M ionomycin of immature oocytes leads to an evident actin cytoskeleton depolymerization in the subplasmalemmal region (Fig. IV 3). Compared to the control eggs in which the cortical granules were closely attached to the plasma membrane by the end of meiotic maturation (Fig. IV 4a, arrowheads, upper panel), in the eggs that had been pretreated with ionomycin before undergoing maturation process the cortical granules were much reduced in number. Hence, as a result of the treatment, the cortical granules located near to the plasma membrane underwent exocytosis (Fig. IV 4a, blue arrows, lower panel) or were engulfed by the white vesicles (Fig. IV 4a, red arrows, lower panel). This was not the case in the non-treated mature eggs, in which cortical granules were intact despite the presence of vicinal white vesicles. Since the ultrastructure of the cortices of the oocytes treated with 5 μ M ionomycin was not altered by further exposure to 1MA (see the TEM images of treated oocytes and eggs in Fig. IV 2c and 4a), the exposure to the ionophore caused irreversible changes in the subplasmalemmal region. Indeed, the actin network subjacent to the plasma membrane was still depolymerized (Fig. IV 3b), and the microvilli were still completely absent in these eggs (Fig. IV 4a). Interestingly, when matured eggs that have been pretreated at the GV stage were again exposed to the same concentration of ionomycin (5 μ M), the intracellular Ca^{2+} increase did not take place (Fig. IV 4b, brown curves), unlike the control non pretreated eggs (Fig. IV 4b, green curves).

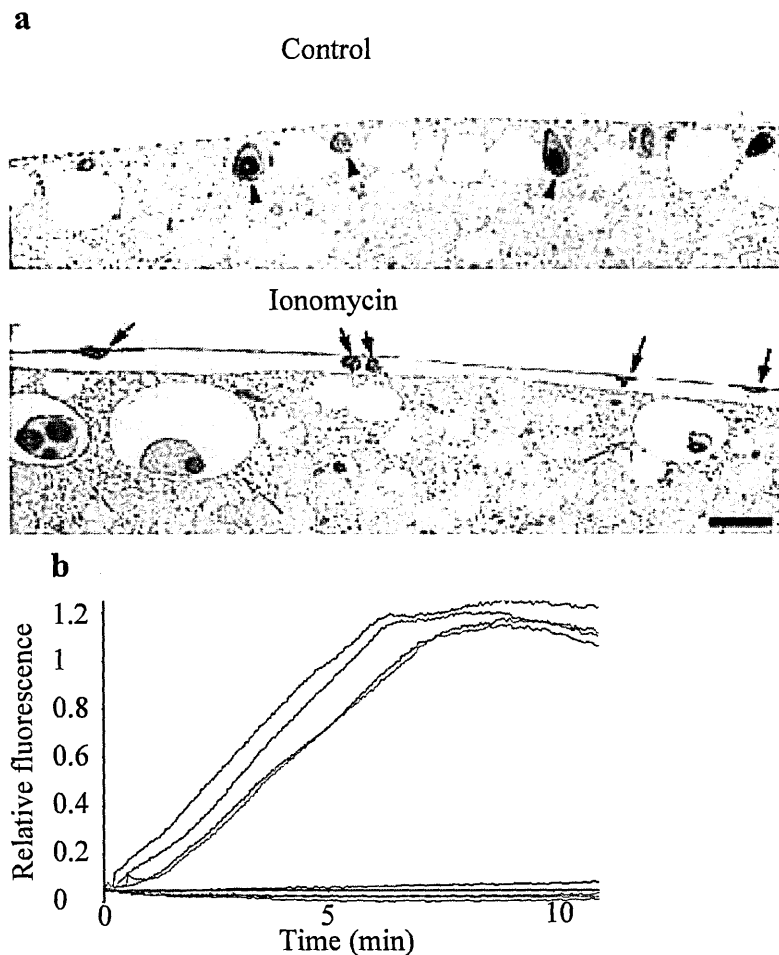


Figure IV 4. Disruption of cortical granules and microvilli by the brief exposure to ionomycin leads to depletion of the ionomycin-sensitive Ca^{2+} stores. *A. aranciacus* GV stage oocytes were treated with 5 μM ionomycin for 3 minutes, washed and stimulated to resume the meiotic cycle by 1-MA. (a) After 1 hour, the mature eggs were fixed with 1% glutaraldehyde and analyzed by TEM. Blue arrowheads indicate the cortical granules attached to the plasma membrane (upper panel), and the remnant of the cortical granules that were extruded in the perivitelline space (blue arrows, lower panel). Red arrows indicate fragments of cortical granules being engulfed by white vesicles. Scale bar, 10 μm . (b) The same batch of oocytes was exposed to 5 μM ionomycin for 3 minutes and transferred to the sea water containing 1-MA. After GV breakdown, the mature eggs were microinjected with Calcium Green / Rhodamine red and subsequently re-exposed to 5 μM ionomycin ($t = 0$) to monitor the Ca^{2+} response. The trajectory of intracellular Ca^{2+} levels in the eggs with or without (control) ionomycin pretreatment were depicted in brown and green curves, respectively.

The same results were obtained with the oocytes from another starfish species *P. miniata*. The brief exposure to 5 μ M ionomycin at the GV stage prevented the mature eggs to increase the intracellular Ca^{2+} after the second exposure after maturation (Fig. IV 5a). The failure of the second exposure to induce a Ca^{2+} response is not due to the potential acidification of the eggs or the inefficacy of ionomycin at low pH. Because ionomycin has no Ca^{2+} -complexing activity below pH 7.0, I used the A23187 ionophore, which reportedly maintains its Ca^{2+} ionophore activity at pH 5.0 to 10 (Liu and Hermann, 1978). Mature eggs exposed to 40 μ M A23187 induced intracellular Ca^{2+} increase, whereas mature eggs pretreated with ionomycin at the GV stage, produced no Ca^{2+} increase (Fig. IV 5b). Taken together, these results indicate that the structural changes caused by the brief ionomycin exposure, i.e. disruption of the cortical granules and the fusion with the white vesicles and elimination of the microfilaments-filled microvilli, are related to the depletion of the ionomycin-sensitive Ca^{2+} stores.

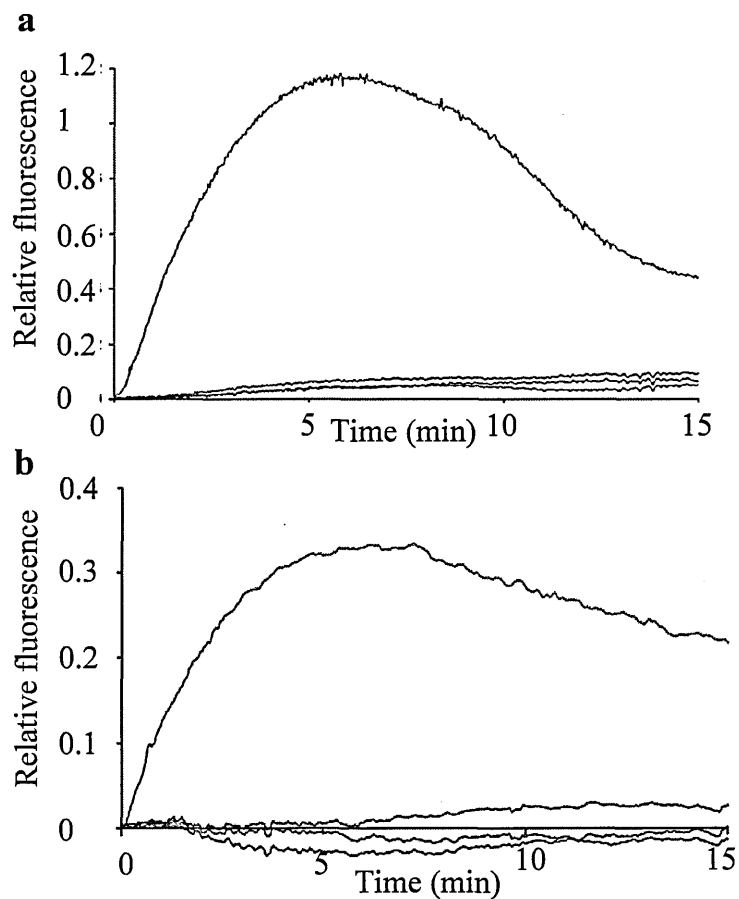


Figure IV 5. The mature eggs pretreated with ionomycin at the GV stage respond to the second dose of ionomycin or A23187 with no intracellular Ca^{2+} increase. *P. miniata* GV stage oocytes were exposed to 5 μ M ionomycin for 3 minutes and transferred to the fresh seawater containing 1-MA (10 μ M) for 1 h and subsequently treated with the second dose of 5 μ M ionomycin (a) or 40 μ M A23187 (b). In both cases, the green curves depict the Ca^{2+} response in the control eggs, and the brown ones the response of the eggs that had been briefly exposed to 5 μ M ionomycin at the GV stage.

Ionomycin-sensitive Ca^{2+} pool in the cortex of starfish eggs

The cytosolic Ca^{2+} increase in immature oocytes induced by the brief exposure to ionomycin had mostly returned or was at least en route to its basal levels one hour after the addition of 1-MA, as was noted in the non-exposed control eggs (data not shown). Even though these eggs do not release intracellular Ca^{2+} to the second exposure to ionomycin and A23187 (Fig. IV 4 and 5), they responded to InsP_3 with a significant Ca^{2+} release (Fig. IV 6a). However, the amplitude of the Ca^{2+} peak in these eggs (0.78 ± 0.23 RFU, $n=10$) was significantly lower (by 43 %) than that of control mature eggs (1.38 ± 0.20 RFU, $n=9$; $P<0.0001$) (Fig. IV 6a and b). Hence, the structural changes in the cortex, e.g. elimination of the microvilli and disruption of cortical granules (Fig. IV 4a), might be linked to the reduction of the InsP_3 -mediated intracellular Ca^{2+} release. Nevertheless, the kinetics of the Ca^{2+} rise induced by InsP_3 in control mature (12.2 ± 4.8 sec, $n=9$) and pretreated eggs (12.54 ± 3.1 sec, $n=10$; $p=0.868$), was not changed, and remained almost the same (Fig. IV 6b). Another characteristic of the ionomycin-pretreated eggs was the failure of the vitelline envelope to elevate despite the substantial intracellular Ca^{2+} release (Fig. IV 6c). In addition, in the majority of cases, repeated insemination was needed for these eggs to produce the characteristic fertilization Ca^{2+} wave (Fig. IV 7a). However, the onset of the Ca^{2+} wave induced by a single sperm in ionomycin pretreated eggs was much delayed (68.7 ± 64.3 sec after sperm addition, $n=3$) in comparison with the control eggs in the same batch of experiment (19.2 ± 9.2 sec, $n=10$; $P<0.05$) (Fig. IV 7a). In parallel, the peak amplitude of the sperm-induced Ca^{2+} increase in the eggs that had been exposed to ionomycin at the immature stage was significantly lowered, by 35.9 % (0.67 ± 0.23 RFU, $n=20$) than that of the control eggs (1.05 ± 0.13 RFU, $n=23$, $P<0.0001$) (Fig. IV 7b). Again, the kinetics of the Ca^{2+} transient was virtually the same, since the time required for reaching the peak in the ionomycin-pretreated eggs (148.4 ± 52.4 sec, $n=20$) was not significantly different from that in control eggs (128.0 ± 26.1 sec, $n=23$, $P=0.1061$) (Fig. IV 7c). Hence, the calcium-releasing mechanism that sustains the propagation of the Ca^{2+}

wave remained intact in these eggs. Nevertheless, the massive and instantaneous sperm-initiated intracellular Ca^{2+} release that takes place beneath the plasma membrane (cortical flash) at the initial moments of the sperm-egg interaction (Santella et al., 2004) was either reduced in its amplitude, or completely abolished in the ionomycin pretreated eggs (Fig. IV 7d). In five independent experiments, the frequency of the cortical flash was highly reduced in ionomycin pretreated eggs ($30 \pm 24 \%$, $n=5$) compared to the control ones ($76 \pm 2 \%$, $n=5$; $P<0.05$) (Fig. IV 7e). In addition, the amplitude of the cortical flash of the pretreated eggs was substantially reduced to merely $47.8 \pm 31.2 \%$ ($n=7$) of the averaged value in the control ($100 \pm 27.6 \%$, $n=17$, $P<0.001$) (Fig. IV 7f). Therefore, these results imply that the fertilization associated events in the cortex of the eggs are highly influenced by the pretreatment of the oocytes with ionomycin.

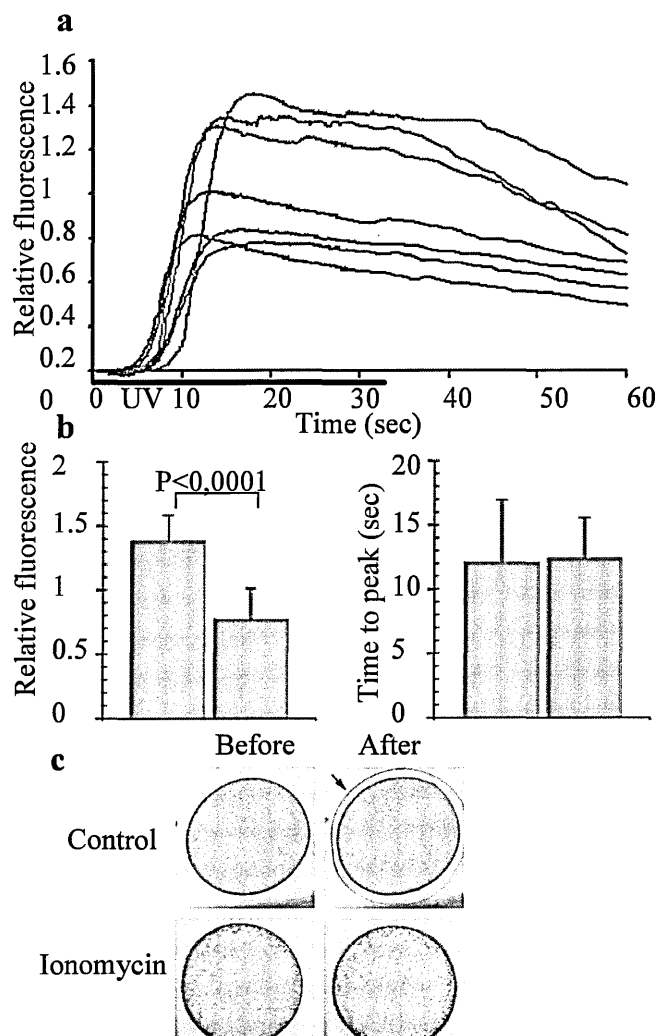


Figure IV 6. Ionomycin-exposed eggs with cortical granule disruption still respond to InsP_3 with an intracellular Ca^{2+} release, but to a reduced extent. *A. aranciacus* oocytes at the GV stage were exposed to 5 μM ionomycin for 5 minutes and microinjected with caged InsP_3 (5 μM) and Calcium Green (500 μM). The oocytes were matured in a seawater containing 1-MA (10 μM) for 1 hour and then irradiated with UV to photoactivate the caged InsP_3 . **(a)** The trajectories of the quantified Ca^{2+} responses at the entire cytoplasmic field are showed for one of the three independent batches of experiments. Ca^{2+} responses in the control eggs (green) and the eggs briefly exposed to 5 μM ionomycin at the GV stage (brown curves) after UV irradiation (violet bar). **(b)** The average amplitude (left histogram) and the time interval between the onset and the peak of the Ca^{2+} signals (right histogram) were depicted of experimental eggs (brown bars, $n=10$) and control (green bars, $n=9$) eggs. **(c)** The experimental eggs did not undergo elevation of the vitelline layer in all cases.

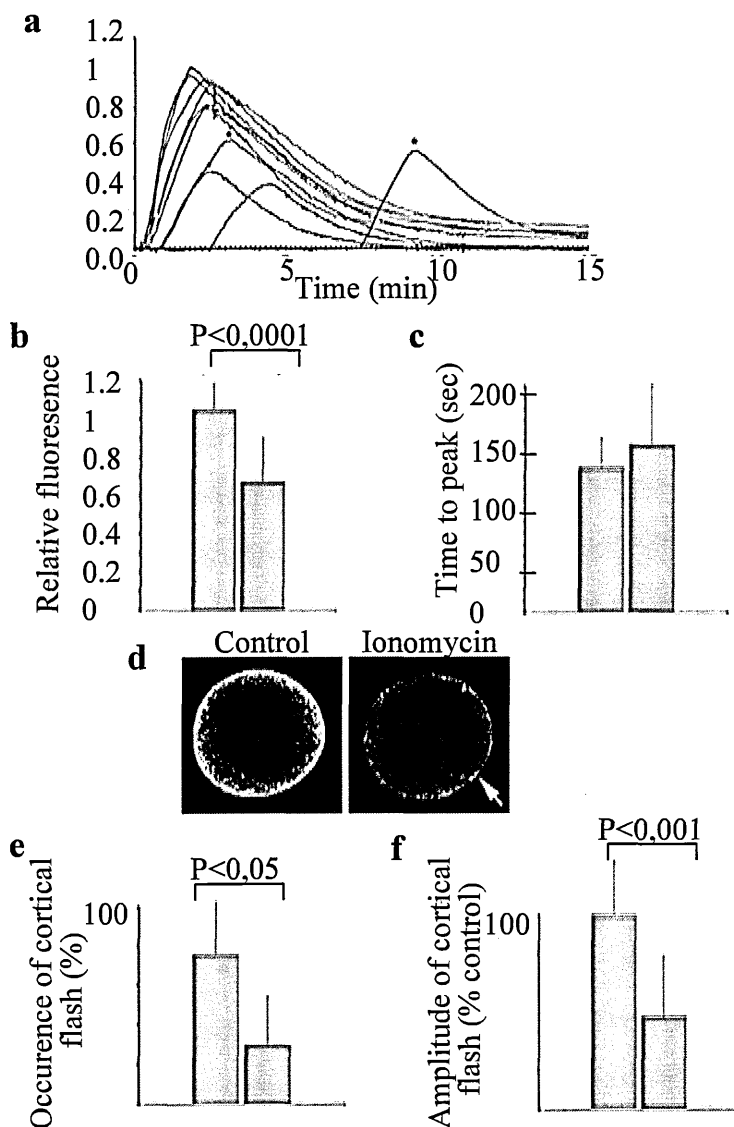


Figure IV 7. Fertilization of the ionomycin-pretreated eggs with altered cortical structure. *A. aranciacus* oocytes were exposed to 5 μM ionomycin (5 minutes) at the GV stage, washed and then exposed to 1-MA (10 μM). Mature eggs were then inseminated. **(a)** Quantification of the intracellular Ca^{2+} levels in control (green) and experimental eggs (brown curves) of one of the five independent experiments. Sperm addition was set to $t = 0$. Asterisks indicate the Ca^{2+} peaks of the eggs that required a second addition of sperm. **(b)** The average amplitude of the Ca^{2+} peaks and **(c)** the time interval between the onset and the peak of the signals in the control (green bars, $n=23$) and experimental eggs (brown bars, $n=20$). **(d)** Pseudocolor images of the representative cortical flashes in the control and the ionomycin-pretreated eggs (arrow). **(e)** Frequency of the detectable cortical flashes. **(f)** Comparison of the amplitude of the cortical flashes. Data were normalized in reference to the average value of the control eggs in each batch of experiment.

Finally, unlike the InsP_3 and the sperm (Fig. IV 6 and 7), the failure of ionomycin to induce intracellular Ca^{2+} release after the second exposure of mature eggs that have been pretreated in the GV stage (Fig. IV 4 and 5), raises the possibility that the ionophore may not diffuse deep into the cytoplasm of big cells like starfish eggs in bath incubation. To examine that, mature eggs of starfish were microinjected with ionomycin. Surprisingly, even the microinjection of control eggs (and not only the ionomycin-pretreated) with 50 μM ionomycin in the injection pipette did not evoke intracellular Ca^{2+} increase. The same negative results were obtained even when 10 times higher ionomycin concentration was used (500 μM , concentration in the microinjection pipette) in both mature and immature oocytes (data not shown). The negative result was not due to a technical problems related to the simultaneous microinjection and Ca^{2+} detection because the InsP_3 microinjections (5 μM in the injection pipette) using the same method produced high levels of Ca^{2+} response in the control eggs (Fig. IV 8).

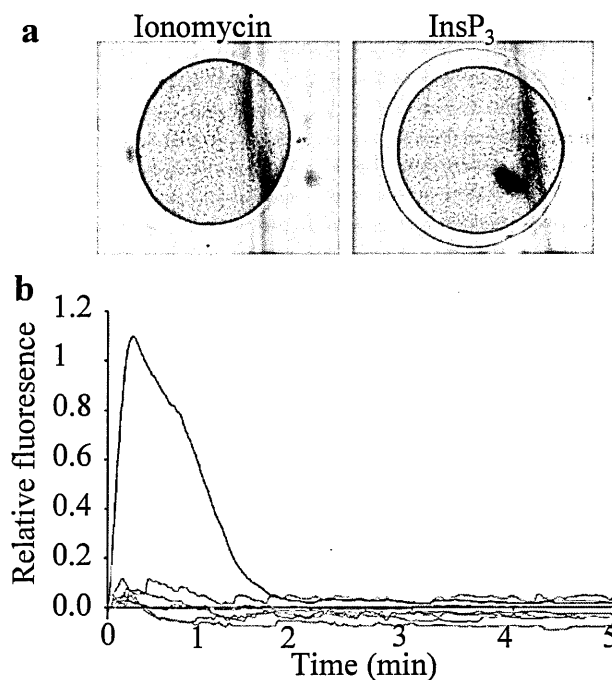


Figure IV 8. Microinjected ionomycin does not induce Ca^{2+} increase inside the starfish eggs. GV stage *P. miniata* oocytes were microinjected with Calcium Green and induced to undergo meiotic maturation for 1 hour in the seawater containing 1-MA (10 μM). Under the CCD camera, the mature eggs were microinjected with InsP_3 (without caging, 5 μM in the pipette tip), ionomycin (50 μM), or the injection buffer only. Results of one of the three independent experiments are shown. (a) Transmission views of the eggs 10 minutes after microinjection of ionomycin (50 μM) or InsP_3 (5 μM). (b) Quantified Ca^{2+} signals for InsP_3 (blue curve), injection buffer (green), and ionomycin (brown).

Fertilization and early development of the ionomycin-pretreated eggs of starfish

Brief exposure of the GV stage oocytes to 5 μ M ionomycin leads to a great loss of cortical granules (Fig. IV 2 and 4). Since cortical granules exocytosis at fertilization is responsible for vitelline layer elevation and fertilization envelope formation known to serve as a slow mechanical block to polyspermy, the eggs pretreated with ionomycin at the GV stage that do not form fertilization envelope were thought to be prone to polyspermic fertilization. To examine that, I have pre-stained the sperm with Hoechst 33342 and counted the number of the sperm that entered the eggs after fertilization. Mature eggs exposed to the same sperm concentration showed mostly monospermic fertilization in the control (86.4 ± 18.8 %, $n=4$ independent experiments). In contrast, insemination of the ionomycin pre-exposed eggs resulted mainly in no sperm entry (75.1 ± 20.5 %, $n=4$) (Fig. IV 9a). Hence, the ionomycin-pretreated eggs revealed much less efficient interaction with the sperm, which leads to the sperm incorporation into the egg. The frequency of monospermic fertilization for the control eggs far exceeds (6.4 fold) the frequency of polyspermic fertilization (13.6 ± 18.9 %, $P<0.001$, Turkey's test in one-way ANOVA). On the other hand, the monospermic fertilization of ionomycin-pretreated eggs (18.5 ± 14.0 %) displayed 3.1 fold higher rate over polyspermy (6.1 ± 8.8 %), but the difference was not statistically significant. Hence, the mechanisms that establish successful monospermic fertilization in the control eggs, seems not to work in the ionomycin pretreated ones. However, the frequency of the polyspermic fertilization in the total egg population in pretreated eggs (20.8 ± 19.1 %, $n=3$ independent experiments) was not significantly different compare to control eggs (16.1 ± 19.6 %, $n=4$, $p=0.7614$). Further, to study the effect of the ionomycin pre-treatment on the early development of the starfish eggs, I followed the fate of the monospermic zygotes. Firstly all the zygotes that have undergone treatment had no thick fertilization envelope formed, in contrast to the control ones (Fig. IV 9b). Secondly, while all zygotes from the monospermic fertilization of the control eggs underwent normal development to the 16 cell stage, four out of eleven pretreated eggs that

clearly showed monospermy at fertilization failed to develop normally at the early stages of the cell cleavage (Fig. IV 9b and c). In particular, they had a problem in creating a clear-cut cleavage furrow and therefore were prone to form amorphous cell clusters (Fig. IV 9b). Finally, in both cases all polyspermic zygotes failed to develop normally (data not shown).

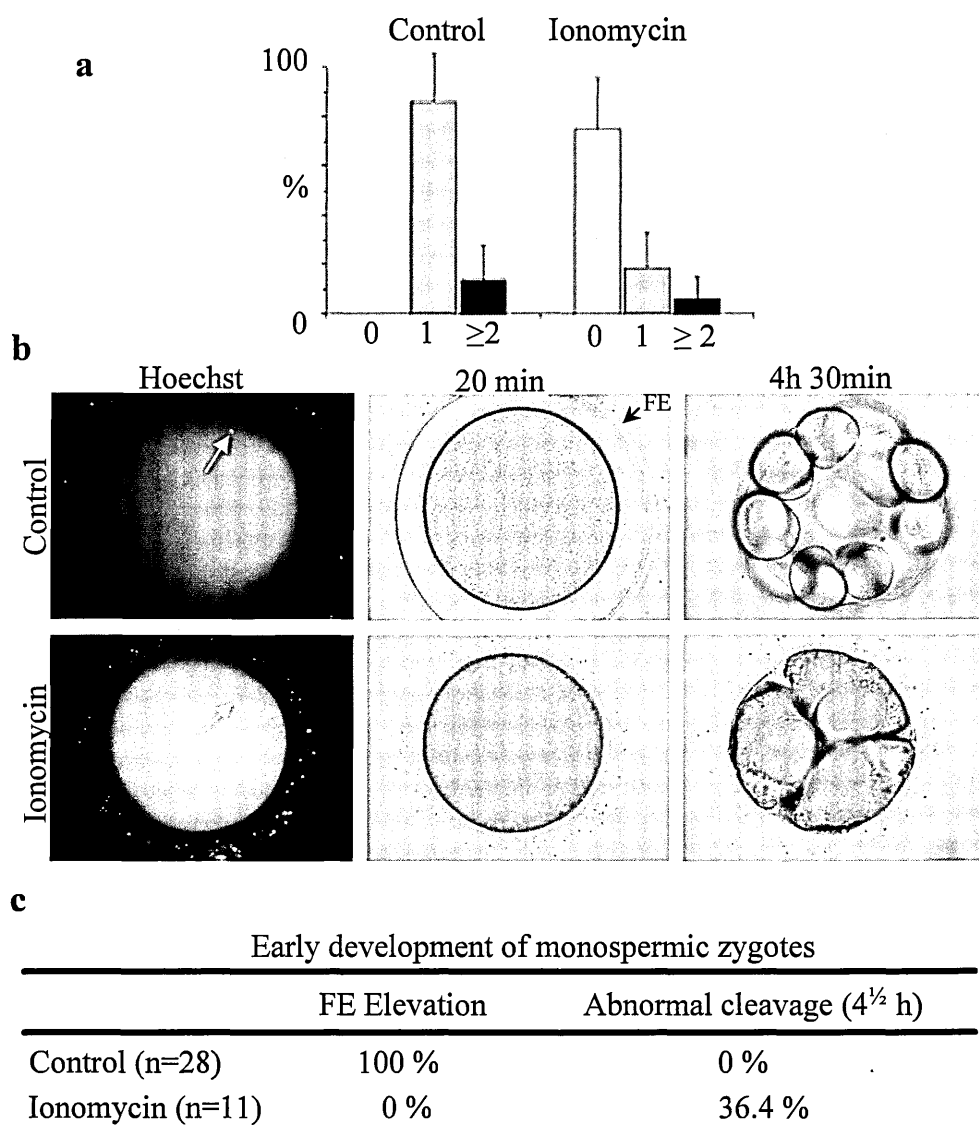


Figure IV 9. Fertilization and the early development of the ionomycin-pretreated eggs. (a) *A. aranciacus* oocytes were pretreated with 5 μ M ionomycin at the GV stage and stimulated with 1-MA for 1 hour. Then, eggs with or without (control) ionomycin pretreatment were fertilized with Hoechst 33342-stained sperm. The number of the internalized sperm in each egg was counted after 20 min of insemination, and the frequencies of monospermy (gray bars), polyspermy (black bars, sperm count >2), or the case with no evident sperm entry (white bar) were calculated in four independent experiments. (b) Developmental progress of the representative control and the ionomycin-pretreated eggs that clearly established monospermic sperm entry (arrows). (c) Summary of the fertilization envelope (FE) formation and the rate of abnormal development in the control and ionomycin-pretreated monospermic zygotes.

Ionomycin pretreatment disrupts the functionality of the cortical actin cytoskeleton at fertilization

Subplasmalemmal actin cytoskeleton is highly implicated in the processes of egg activation at fertilization. The actin cytoskeleton, besides the intracellular Ca^{2+} rise, is a decisive factor controlling exocytosis of vesicles, as suggested in various cell types (Muallem et al., 1995; Gasman et al., 2004; Malacombe et al., 2006; Kyozyuka et al., 2008; Puppo et al., 2008; Chun et al., 2010). Despite the relatively high intracellular Ca^{2+} increase induced by the sperm and InsP_3 , the elevation of the vitelline envelope in ionomycin pretreated eggs was blocked (Fig. IV 6 and 8), indicating strong interference with the egg activation processes. In the fertilized eggs of echinoderms, it was demonstrated that the subplasmalemmal actin fibers migrate centripetally toward the inner cytoplasm simultaneously with the elevation of the fertilization envelope (Terasaki, 1996; Puppo et al., 2008; Chun et al., 2010). In order to investigate the cause of the ineffective sperm entry and the abnormal cell cleavage in the ionomycin-pretreated eggs, I surveyed whether or not the mobilization of the actin cytoskeleton in fertilized eggs is ever affected by the ionomycin pretreatment (Fig. IV 10). In contrary to the control eggs, in which the cortical actin fibers underwent centripetal movement toward the egg's center, in the ionomycin pretreated eggs neither such coordinated translocation occurred, nor the vitelline envelope elevated (Fig. IV 10). Hence, the ionomycin pretreatment of the oocytes not only altered the actin cytoskeleton structure but also affected its functionality.

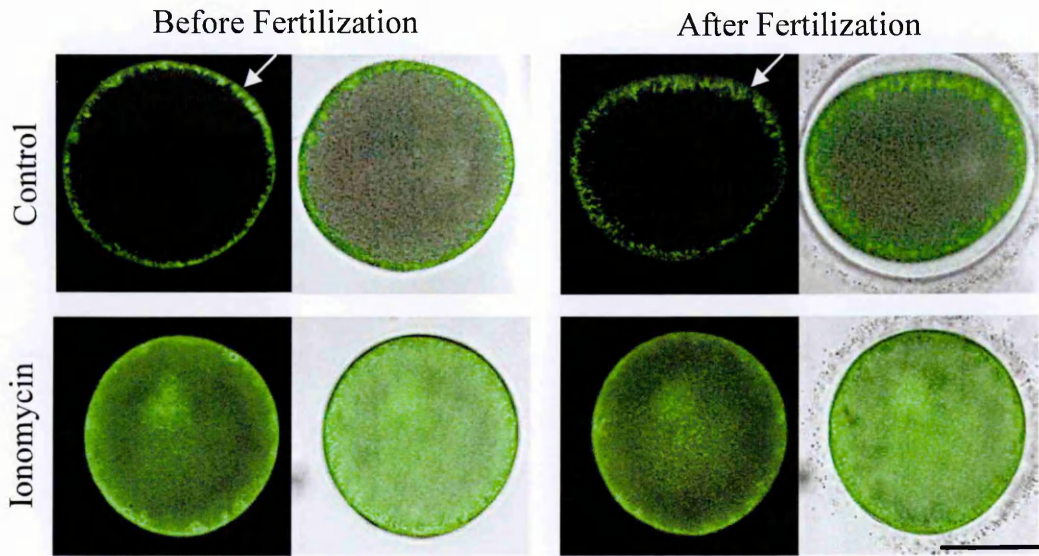


Figure IV 10. Ionomycin pretreatment disrupts the functionality of the cortical actin cytoskeleton. *P. miniata* GV stage oocytes were exposed to 5 μ M ionomycin for 8 minutes, washed and then stimulated for 1 hour with 1-MA (10 μ M) to undergo meiotic maturation. After 1 hour, the mature eggs were microinjected with Alexa-Fluor 488-conjugated phalloidin. The real-time changes in the actin cytoskeleton (arrows) were monitored with confocal microscopy before and after insemination. At the right side of each panel, the fluorescence image of F-actin in confocal microscopy was merged with the transmission view of the same specimen. Images of the same individual eggs were taken before and after fertilization (13 minutes post-insemination). Scale bar, 100 μ m.

Deleterious effects of ionomycin on development

In order to study the effect of ionomycin on early development of the monospermic zygotes, I screened for the eggs that had been fertilized by a single sperm and that clearly demonstrated full elevation of the fertilization envelope. Then, these monospermic zygotes were exposed to 5 μ M ionomycin, the similar dose in which the human and animal eggs are exposed to induce egg's activation in the practice of intracytoplasmic sperm injection (ICSI) (Terada et al., 2009; Heytrens et al., 2010). The data pooled from three independent experiments showed that most of the control zygotes developed normally 4 hours after the fertilization (88.3 ± 1.4 %), whereas only 24.8 ± 10.9 % of the monospermic zygotes treated with ionomycin developed normally at 4 hours ($P < 0.001$) (Fig. IV 11a). These monospermic zygotes exposed to ionomycin displayed either blocked first cell division or a problem in cell cleavage at later stages (Fig. IV 11a). After 3 days of the given experimental condition, the control embryos developed normally in majority of the cases (68.3 ± 6.1 %), while the monospermic zygotes exposed to ionomycin after fertilization displayed significantly reduced rate (16.2 ± 19.3 %, $P < 0.05$) of the normal progression of the development (Fig. IV 11b).

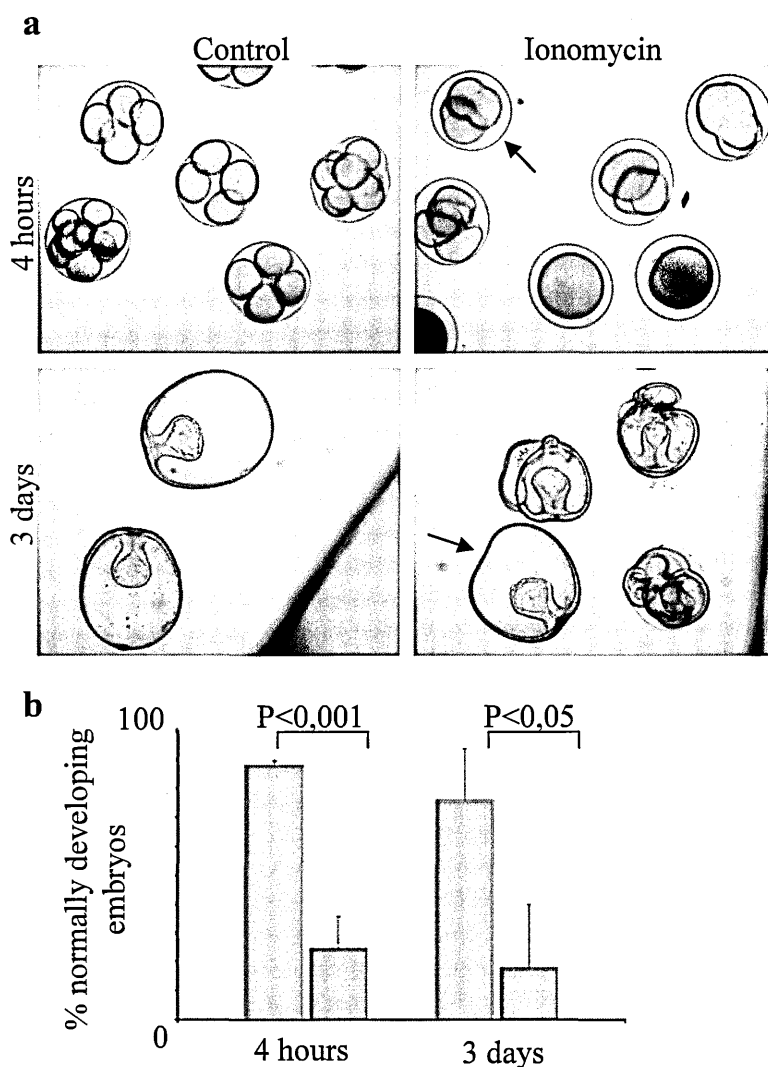


Figure IV 11. Deleterious effects of ionomycin on development. Mature *A. aurantiacus* eggs were fertilized with the Hoechst 33342-stained sperm, and after 20 minutes monospermic zygotes displaying fully elevated fertilization envelope were exposed to 5 μ M ionomycin or to 0,1 % DMSO (control, vehicle) for 10 minutes. Further, the zygotes were incubated in seawater to follow the progress of development. (a) Representative photomicrographs of the early embryos developing from the control and the monospermic zygotes exposed to ionomycin. Normally developing zygotes that have been exposed to ionomycin are indicated with arrows at 4 hours and 3 days after fertilization. (b) Frequency of normal development in the control and the ionomycin-exposed zygotes. Data were pooled from three independent batches of experiments comprising 8 to 10 monospermic zygotes with (brown bars) or without (control, green bars) the 10 min ionomycin treatment after fertilization.

Discussion for Chapter IV

In this chapter, I have studied the effects of ionomycin on egg activation and subsequent development in starfish. Ionomycin has been widely used in the laboratories studying Ca^{2+} signaling, and has also been used as a part in the standard protocol to activate the eggs that do not respond to ICSI in *in vitro* fertilization method (Terada et al., 2009; Heytens et al., 2010; Ciapa and Arnoult, 2011). Given that ionomycin by itself activates mature eggs, I have used the GV stage oocytes to briefly expose them to the ionophore and follow the meiotic maturation, fertilization and early development. Treatment of oocytes with 5 μM ionomycin induces significant intracellular Ca^{2+} increase (Fig. IV 1b). Owing to the large cell size, I was able to follow the spatiotemporal changes of the intracellular Ca^{2+} levels during the ionomycin treatment. First, I have shown that during the ionomycin treatment there is a significant contribution of the influx to the Ca^{2+} rise. The peak of the intracellular Ca^{2+} in the CaFSW was merely 53.4% of the level in the Ca^{2+} -containing seawater, indicating for a substantial contribution of the influx to the Ca^{2+} rise (Fig. IV 1). However, with the meiotic maturation the intracellular Ca^{2+} rise in CaFSW was as 79.0% (0.60 ± 0.18 RFU, $n=11$) of that in the ASW (0.76 ± 0.12 RFU, $n=11$) containing 10mM Ca^{2+} , implying a higher contribution of the internal stores to the net intracellular Ca^{2+} increase by ionomycin.

By incubating the oocytes with ionomycin, I have shown that there was a rapid increase in the intracellular Ca^{2+} which was accompanied by a striking reorganization of the actin cytoskeleton network in the distinct subcellular domains (Fig. IV 3a). The molecular mechanism underlying these cytoskeletal changes and their physiological significance are yet to be known. Since there is a class of actin-binding protein, e.g. gelsolin, that becomes functionally active to remodel cytoskeleton when binding to free Ca^{2+} (Burtneck et al., 2004), it is conceivable that similar pathway may be at work to sever actin filaments or increase the plus ends of actin filaments. Considering that actin is the most abundant (up to 300 μM) Ca^{2+} -binding protein in the cytosol with a high binding activity ($K_d = 2\text{-}8 \times 10^{-9}$

M for ATP-bound G-actin) (Carlier et al., 1986; Gershman et al., 1986), and that the Ca^{2+} ions, once bound and buried in the groove of F-actin, are inaccessible for exchange (Kasai and Oosawa, 1969), the formation of the F-actin in the cell compartment with elevated Ca^{2+} levels could serve as a mechanism to absorb and store Ca^{2+} ions. The extensive hyperpolymerization of actin due to the ionomycin treatment might thus alleviate at least in part the cytosolic Ca^{2+} increase (Lange and Gartzke, 2006; Santella and Chun 2011). Hence, it may function as a cell's adaptive defence mechanism against the potential toxicity of prolonged Ca^{2+} rise. On the other hand, actin cytoskeleton depolymerization in the cortex may be involved in the regulation of store-operated Ca^{2+} entry that was shown to occur in ionomycin-treated cells (Morgan and Jacob, 1994; Darbellay et al., 2011).

It was also shown that the rapid actin cytoskeleton rearrangement was accompanied by the formation of the large white vesicles, which are often vested with actin filaments (Fig. IV 3b). This type of vesicles appeared with the concomitant loss of smaller vesicles of the same morphology which were always present in the subplasmalemmal region of control oocytes. Since the intermediate twined structure resembling two white vesicles at fusion (Fig. IV 2c) seen in the ionomycin-treated oocytes but not in the control ones, it was concluded that the large white vesicles are formed by fusion with other vesicles. Being often observed inside the white vesicles, the electron-dense materials are likely to be fragments of the cortical granules (Fig. IV 4a, red arrows, lower panel). The physiological role of the large white vesicles is unknown, but their morphology observed in ionomycin-pretreated oocytes is reminiscent of the 'clear granules' containing residual electron-dense materials in the normal sea urchin eggs of *Arbacia punctuate* (Ramos et al., 2010). These clear granules (0.83 μm in diameter) in the sea urchin eggs were found to contain large amount of Ca^{2+} and polyphosphate and were identified as 'acidocalcisome'-like organelles. While the formation of the large white vesicle could be a result of a cell stress due to a dramatic actin cytoskeleton rearrangement and extended intracellular Ca^{2+} increase in the

ionomycin-pretreated eggs, the physiological role of the ‘small’ white vesicles seen in the control eggs is still to be resolved.

A brief ionomycin treatment of immature oocytes leads to a intracellular Ca^{2+} increase and to the loss of cortical granules that are partially exocytosed or engulfed by the white vesicles (Fig. IV 2 and 4). Interestingly, when the ionomycin-pretreated oocytes were induced to undergo meiotic maturation by addition of 1-MA and then exposed to the same dose of ionomycin, they did not show any increase of the intracellular Ca^{2+} (Fig. IV 4b), raising the possibility that the loss of the cortical granules may be mainly responsible for the ionomycin-sensitive Ca^{2+} increase. This result is in agreement with the early studies in which *Xenopus* oocytes did not produce Ca^{2+} -dependent Cl^- currents in response to the second exposure to ionomycin if the oocytes had been previously treated with the suprathreshold (1 μM) dose of ionomycin (Yoshida and Plant, 1992). In *Xenopus* oocytes this refractory period could be overcome by washing the oocytes for 50 minutes, whereas the starfish eggs in this study did not show any sign of recovery from the refractory phase even after 70 minutes after removal of the ionomycin from the media. Hence, I was not able to establish whether this refractory period is reversible in starfish eggs. Despite the apparent loss of the responsiveness to ionomycin, the eggs of the same treatment (5 minutes exposure to ionomycin at the GV stage) still responded to InsP_3 and sperm with the substantial intracellular Ca^{2+} increase (Fig. IV 6 and 7), although with the consistently lower amplitude than in the ionomycin-untreated eggs. A possible explanation for this result is that the internal Ca^{2+} stores sensitive to InsP_3 and sperm were not fully recharged after the ionomycin treatment. Alternatively, we can not rule out the possibility that the optimization of the intracellular Ca^{2+} stores that occurs during oocyte maturation (Chiba et al., 1990; Lim et al., 2003) might have been interfered with as a result of the ionomycin pretreatment. Finally, given that the pretreated eggs fail to respond to the second exposure to ionomycin and A23187 (Fig. IV 5), the reduction of the Ca^{2+} response may represent a fraction of the internal Ca^{2+} stores that is sensitive to ionomycin and InsP_3 but had been

destroyed by the ionomycin pretreatment. However, from these data it is difficult to locate which structure represents the ionomycin-destroyed internal Ca^{2+} store sensitive to InsP_3 . While the conventional ER Ca^{2+} store may be affected by the treatment, it is conceivable that the loss of microvilli, cortical granules and white vesicles may contribute to the InsP_3 -induced Ca^{2+} increase. In line with the idea that the cortical granules represent the major cortical Ca^{2+} stores, accounting for nearly 10 % of total Ca^{2+} storage in sea urchin eggs (Gilot et al., 1991), the reduction in the InsP_3 -dependent Ca^{2+} release may be ascribed to the loss of the cortical granules or their associated structures. However, the Ca^{2+} contained in the cortical granules is not likely to contribute to the intracellular Ca^{2+} increase at fertilization. Secretory vesicles of any types seem to lack InsP_3R (Rizzuto and Pozzan, 2006), and if cortical granules do not have Ca^{2+} -releasing channels on their membranes, then the luminal Ca^{2+} would be extruded in the perivitelline space during exocytosis at fertilization. Nevertheless, according to the early studies, cortical granules are tightly connected with fine network of ER on the membrane of which reside ryanodine receptors (McPherson et al., 1992). Hence, if they also contain InsP_3R , then the reduced InsP_3 -sensitive Ca^{2+} response that contributes to the Ca^{2+} signaling at fertilization could be from the ionomycin-disrupted endoplasmic cisternae ensheathing the cortical granules, and not the cortical granules proper. However, the ER-enriched microsomal fraction of sea urchin egg cortices sometimes do not respond to InsP_3 with the detectable Ca^{2+} release (Oberdorf et al., 1986). Therefore, the potential contribution of the Ca^{2+} stored in the cortical granules and in the associated structures to the intracellular Ca^{2+} increase at fertilization still remains as an open question. An alternative explanation of the diminished intracellular Ca^{2+} increase in response to InsP_3 and sperm can be that InsP_3 -dependent Ca^{2+} -releasing mechanism may have a reduced efficacy following the dramatic alteration of the actin cytoskeleton structure, as has been suggested in the starfish oocytes and eggs (Nusco et al., 2006; Kyoizuka et al., 2008; Puppo et al., 2008; Chun and Santella, 2009).

The results in this chapter raise a question about how ionomycin works inside the cell. It may diffuse inside the cell and target all the Ca^{2+} stores to transport Ca^{2+} across the membrane and down the concentration gradient. However, ionomycin showed to have specific targets in exerting its impact, because at the same profundity of the egg cytoplasm the membrane fusion was restricted only to cortical granules and white vesicles, while yolk platelets and other organelles were not disrupted or modified by the treatment. One possible explanation for that could be that the vesicles specialized in secretion, e.g. cortical granules and white vesicles (Vasilev et al., 2012) might specifically possess a Ca^{2+} -sensing membrane-bound proteins that interact with cytoskeleton and facilitate membrane fusion, as demonstrated by synaptotagmin in synaptic vesicles (Lee et al., 2010). Since white vesicles are often delineated by fluorescent probes for F-actin (Fig. IV 3b), it is possible that prolonged intracellular Ca^{2+} rise by ionomycin have provoked deregulated membrane fusion between these vesicles in a mechanism that involves actin cytoskeleton. Furthermore, given that ionomycin functionality highly depends on pH (Liu and Hermann, 1978), such specificity might be associated with the subtle difference in the luminal pH of these vesicles and organelles (Morgan, 2011).

Mature eggs that had been pretreated with ionomycin at the GV stage responded with Ca^{2+} increase to InsP_3 but not to the second exposure to ionomycin. That was not due to the possible problems of ionomycin in diffusing across the eggs, since microinjection of ionomycin in the center in the egg cytoplasm did not induce any Ca^{2+} rise (Fig. IV 8). This data suggest that in order to evoke intracellular Ca^{2+} increase, ionomycin should be added to the cells from the side of the higher Ca^{2+} concentration (to the media), as it is always done in the laboratory practice. Alternatively, Ca^{2+} ions transported inside the cell from outside by ionomycin could sustain the further intracellular Ca^{2+} increase in an indirect way, by inducing CICR or activating Ca^{2+} -sensitive enzymes such as PLC. Nevertheless, in an assay using PH-GFP, I could not observe any sign of hydrolysis of PIP_2 in the plasma membrane (data not shown), which is at variance with the results obtained in fibroblast

(Van Rheenen and Jalink, 2002) and starfish eggs at fertilization (Chun et al., 2010). Thus, the question of how ionomycin works inside the cells merits further investigation also in other experimental models.

Finally, the pretreatment of immature oocytes with Ca^{2+} ionophore ionomycin was shown to have detrimental effects on both egg activation and the early embryonic development (Fig. IV 9, 10 and 11). Ionomycin-pretreated oocytes were still able to undergo meiotic development at least in the nucleus, but, they revealed several problems at fertilization. Firstly, these eggs were not able to establish an effective interaction with the sperm at fertilization. Given that the production of the initial Ca^{2+} spot underneath the plasma membrane at the site of the sperm-egg contact is a sign of a successful interaction of the gametes (Santella et al., 2004), the initiation of the Ca^{2+} waves in the ionomycin-pretreated eggs required more than three times longer latent period compare to control (Fig. IV 7a). The microvilli may play an important role in the gametes interaction (Runge et al., 2007), and in this study I found them largely disrupted on the surface of the ionomycin-pretreated eggs (Fig. IV 2 and 4). Then, because of the loss of the cortical granules and other secretory vesicles (Fig. IV 2 and 4) in the ionomycin-pretreated eggs, the fertilization envelope failed to elevate in all cases (Fig. IV 9b). Furthermore, in these fertilized eggs, the centripetal migration of the cortical actin filaments that takes place during the egg's activation was completely abolished (Fig. IV 10). Hence, the ionomycin-pretreated eggs at the GV stage lacked several important features of the normal fertilization process in echinoderms. The monospermic zygotes derived from these fertilized eggs showed further abnormality during development. While the control monospermic zygotes 4.5 hours after insemination showed normal progression of division beyond the 4-16 cells stage, the monospermic zygotes from ionomycin-pretreated eggs displayed unsuccessful cell cleavage (Fig. IV 11a and b). Whether the failure of the monospermic zygotes derived from the ionomycin-pretreatment to proceed with the normal development is due to a failed formation of the fertilization envelope (which is thought to be protective of the

developing embryo) or to a dramatic alteration of the egg ectoplasm (Fig. IV 2 and 4) is a matter of discussion. However, it is important to note that the zygote with a fully elevated fertilization envelope, when exposed to the same dose of ionomycin (5 μ M), displayed a comparable failure rate for cell cleavage and development (Fig. IV 11). Thus, the detrimental effect of ionomycin on embryo development is likely to be caused by the changes in the egg ectoplasm rather than the failure of the eggs to form the fertilization envelope. There might be several different factors responsible for this, but I focused on the actin cytoskeleton. Indeed, actin cytoskeleton is implicated in many cellular events at fertilization and early development (Santella and Chun, 2011). In sea urchin eggs, the cell cleavage of zygotes is highly dependent on the regulation of the actin cytoskeleton (Dale and De Santis, 1981). As it was recently demonstrated, the actin filaments in the egg cortex extend through the perivitelline space of the fertilization envelope in starfish (Chun et al., 2010). Hence, the actin cytoskeleton is closely implicated in the fertilization-associated events and in the early stages of development. Although it is difficult to make a direct comparison between the results of this study and the similar use of ionomycin in the rare case of the medical practice of ICSI in the *in vivo* fertilization clinics (Nasr-Esfahani, 2008), the results of this study call for a more detailed studies on the safety of such procedure used for *in vitro* fertilization of human eggs.

CHAPTER V

Roles of the egg actin cytoskeleton in the regulation of monospermic fertilization in starfish

Results

The actin cytoskeleton may be linked to the regulation of sperm entry: experiments on starfish eggs pretreated with nicotine and BAPTA

In sea urchin and starfish eggs, it is well known that the actin cytoskeleton in the subplasmalemmal region undergoes centripetal migration towards the inner cytoplasm at fertilization during the vitelline envelope elevation (Terasaki, 1996; Puppo et al., 2008; Chun et al. 2010). If this migration is blocked, the sperm often fails to enter the egg (See in Chapter IV Fig. IV 9a and IV 10; Vasilev et al., 2012). At the time of the sperm entry, a specialized structure called ‘fertilization cone’ is formed on the egg’s surface that serves to engulf the sperm. The fertilization cone is filled with the F-actin that is continuous with the long filamentous actin bundles inside the cytoplasm, which appeared to be associated with the penetrating sperm (Puppo et al 2008; Chun et al., 2010). Concurrent with the elevation of the vitelline layer, the spikes protruding from the plasma membrane and traversing the perivitelline space was also shown to be filled with microfilaments (Chun et al., 2010). Hence, the actin cytoskeleton in the cortex of the egg is highly implicated in the processes of fertilization. Interestingly, by perturbing the cortical actin cytoskeleton structure in the starfish eggs by various means, e.g. heparin, JAS and sequestration of plasma membrane PIP₂, there were signs of multiple sperm interactions and entries (Puppo et al., 2008; Chun et al., 2010). In heparin pre-incubated eggs, an abnormal formation of fertilizing cones were observed, whereas JAS-exposed eggs failed to undergo normal elevation of the fertilization envelope despite the comparably high intracellular Ca²⁺ increase and many sperm-egg interactions. Thus, it appears that the fine regulation of the actin cytoskeleton that normally occurs at the egg cortex during fertilization appears to play a pivotal role in

guiding sperm entry and cortical granules exocytosis. Therefore, it was of interest to study how the regulation of the state of the actin cytoskeleton might contribute to ensure monospermic fertilization.

Since the first discovery that nicotine induces polyspermic fertilization (Hertwig and Hertwig, 1887) it has been widely used to study the mechanisms controlling sperm entry because the treatment provides more chance to observe egg-penetrating sperm (Rothschild and Swann, 1950; Hagstrom, 1956; Longo and Anderson, 1970). Firstly, I have confirmed the previous data of others (Rothschild 1952; Longo and Anderson, 1970; Dale, 1985) that nicotine treatment of mature eggs induces polyspermic fertilization (Fig. V 1). As shown in Fig. V 1a, 40 minutes exposure of mature eggs to nicotine (300 μ M) induced high polyspermic fertilization, as judged by the number of Hoechst 33342 (a fluorescent DNA dye) pre-stained sperm that entered the eggs. In contrast, monospermic fertilization was observed in most untreated control eggs. Nicotine treatment rendered the starfish eggs more susceptible to supernumerary sperm entry (40.5 ± 33.6 , $n=6$) compared to the control eggs (1.17 ± 0.41 , $n=6$, $P<0.05$) (Fig. V 1b). In addition, while the vitelline layer elevation occurred in all control eggs ($n=6$), a nicotine pretreatment affected the cortical granules exocytosis and fertilization envelope formation in all studied eggs ($n=10$), by showing either partial ($n=5$) or complete blockade ($n=5$) (Fig. V 1c). These results suggest that a failure in the fertilization envelope elevation, the so called mechanical slow block to polyspermy may be the reason for the high number of sperm entry. As previously shown (Kyoizuka et al., 2008; Puppo et al., 2008), the failure of the vitelline envelope elevation often implies a possible deregulation of the cortical actin cytoskeleton. Therefore, to gain more insights of the role the actin cytoskeleton might play in regulating the block to polyspermy in *A. aranciacus* starfish eggs, I have examined whether or not the nicotine treatment ever changes the structure of the actin cytoskeleton in the eggs. To this end, the nicotine-pretreated eggs were microinjected with Alexa 568-conjugated phalloidin (50 μ M, pipette concentration) and monitored for the changes in the actin-cytoskeleton

structure under the confocal microscope. Indeed, confocal microscopy images taken few minutes after the microinjection revealed a dramatic rearrangement of the actin cytoskeleton underneath the plasma membrane (Fig. V 2). It appears that the actin fibers in the cortex were shifted from the very subplasmalemmal zone to form polymerized bundles in the more inner cytoplasmic regions (Fig. V 2b and b'). This striking actin cytoskeleton reorganization observed after 40 minutes of nicotine (300 μ M) treatment was completely absent in the time-matching control eggs (Fig. V 2a and a'), in which the phalloidin-stained actin fibers exhibited the characteristic perpendicularly orientation immediately beneath the plasma membrane. Since the fluorescent phalloidin probe was microinjected after the nicotine treatment and was used at low dose that does not influence the normal progression of the dynamic movement of the actin fibers (Kyojuka et al., 2008; Chun et al., 2010), these observation in the nicotine pretreated eggs were not caused by the potential actin-stabilizing effect of the phalloidin probe itself.

In addition, another agent used to study the Ca^{2+} signaling at fertilization, the calcium chelator BAPTA, which buffers the intracellular Ca^{2+} increase induced by the sperm, induces similar effect on the cytoskeleton structure in starfish eggs (Fig. V 3b). It was already demonstrated that microinjection of BAPTA into the mouse (Halet et al., 2002; McAvey et al., 2002) and sea urchin eggs (Turner et al., 1986; De Simone et al., 1998) completely blocks or delays the sperm-induced intracellular Ca^{2+} rise and consequently the egg activation at fertilization. Since the exocytosis of the cortical granules is important to provide a mechanical block to polyspermic fertilization in echinoderms and is dependent on the proper actin cytoskeleton organization (Longo et al., 1995; Santella et al., 1999; Puppo et al., 2008), I have studied whether the BAPTA preinjected starfish eggs could alter its dynamics. As shown in Fig. V 3 on the confocal and merged image (transmission light and confocal images), the phalloidin staining 20 minutes after the microinjection of the eggs with BAPTA (100 mM, pipette concentration) produced a drastic reorganization of the actin cytoskeleton in the cortex (Fig. V 3b and

3b') that was strikingly similar to the changes induced by nicotine (Fig. V 2b). In contrast, the time-matching control eggs microinjected with the injection buffer again displayed the expected characteristic structure of the actin cytoskeleton (Fig. V 3a and 3a'). These observations raise an intriguing possibility that the structural alteration of the actin cytoskeleton at the plasma membrane region of the eggs might be the cause of the supernumerary sperm entry. To test that, BAPTA-treated eggs were inseminated with the Hoechst 33342-prestained sperm and after 15 minutes observed under the CCD camera to count the number of the sperm that entered the eggs. As shown on the transmission light images, the BAPTA pre-injected egg failed to elevate the vitelline envelope, which was not the case in the control egg (Fig. V 4a). In parallel with the similarities in the structural changes of the actin cytoskeleton in the BAPTA-microinjected and the nicotine-pretreated eggs, the BAPTA-preinjected eggs showed higher number of successful sperm entry (53.8 ± 48.1 , $n=16$) (Fig. V 4b and c) as was observed in the nicotine-treated eggs (Fig. V 1b). In the same time frame, 76.2 % (16 eggs out of 21) of control eggs were monospermic, in which in average 1.28 ± 0.56 successful sperm entry was detected ($P<0.0001$) (Fig. V 4b).

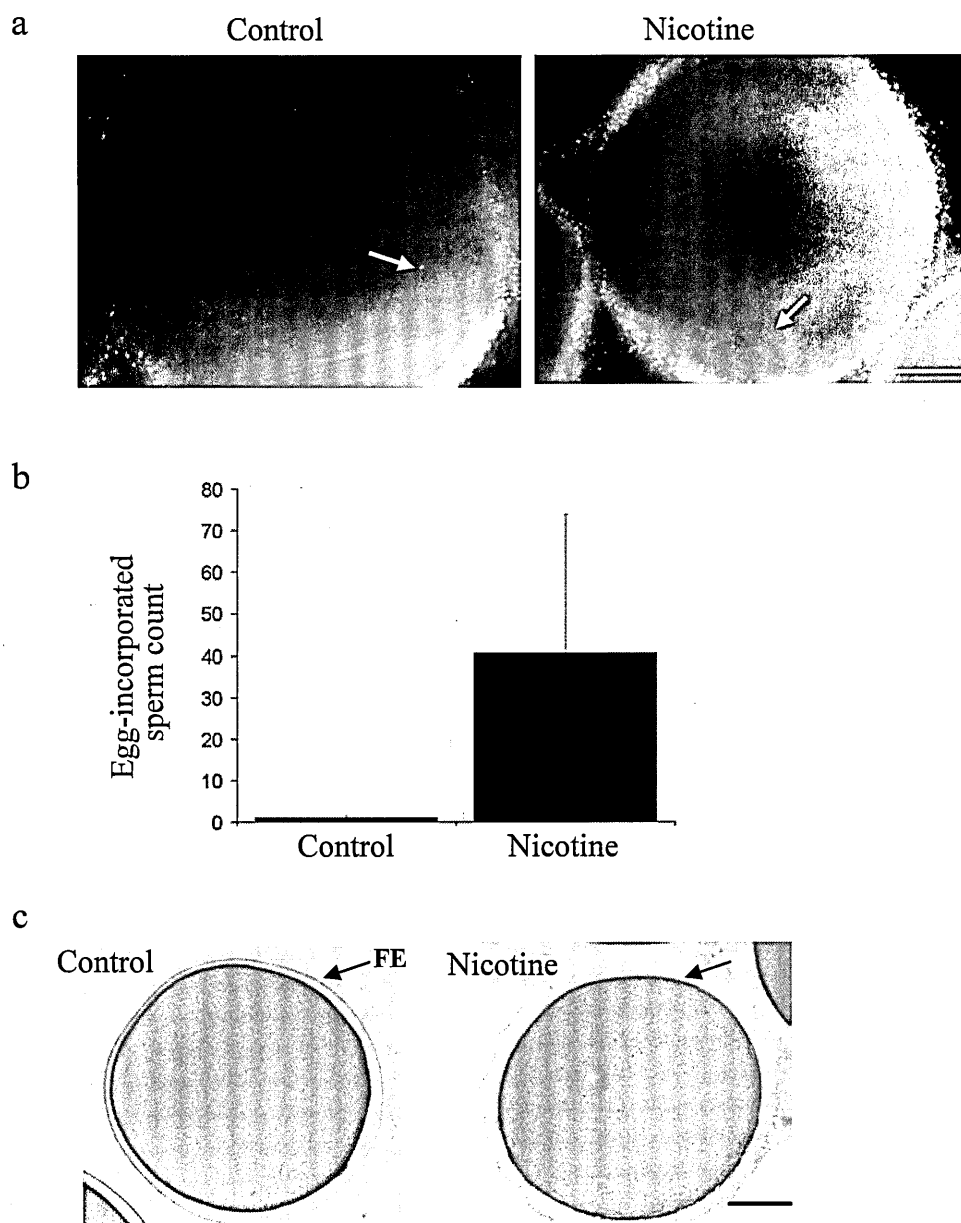


Figure V 1. Nicotine induces polyspermic fertilization in *A. aurantiacus* eggs. (a) Mature eggs treated with nicotine (300 μ M) for 40 minutes, were exposed to Hoechst 33342-prestained sperm in the presence of nicotine (300 μ M), and monitored on a CCD camera. Eggs-incorporated sperm were visualized with UV light (330 nm). Treated mature eggs showed increased sperm entry (right panel, arrow), while the untreated control eggs showed only one sperm incorporated (left panel, arrow). Scale bar, 50 μ m. (b) Histogram displaying the sperm entry number in control (1.17 ± 0.41 , n=6) and experiment eggs (40.5 ± 33.6 , n=6; $P < 0.05$). (c) Transmission light images of fertilized control and nicotine pretreated eggs (FE-fertilization envelope). Scale bar, 50 μ m.

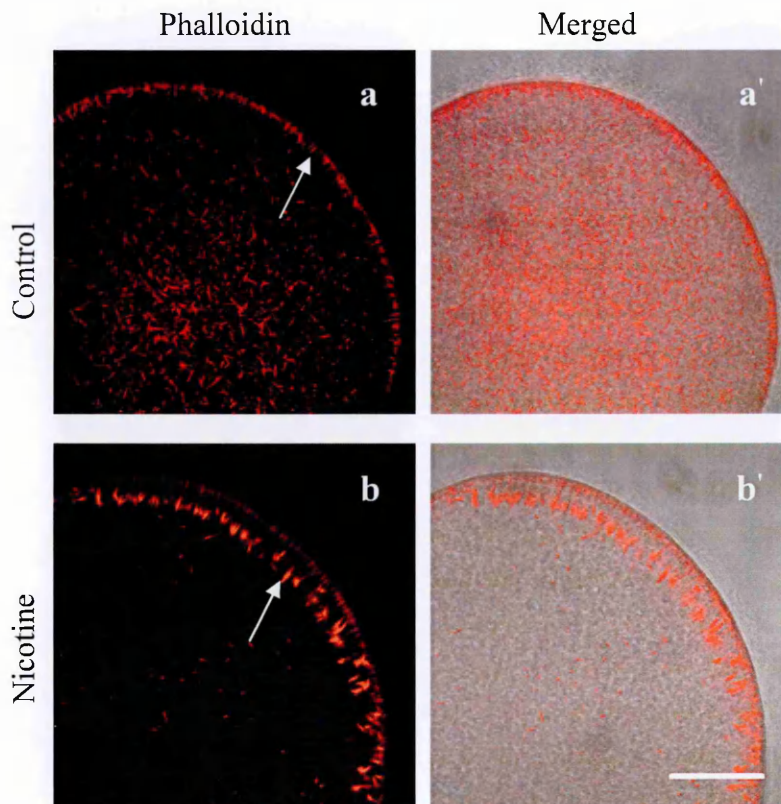


Figure V 2. Nicotine treatment alters the cortical actin cytoskeleton in mature eggs.

(a) Confocal microscopy image of the control mature egg stained with the Alexa 568-conjugated phalloidin (50 μ M), and (a') merged (transmission light and confocal) image of the same egg. The eggs were incubated in fresh seawater for 40 min prior the acquisition.

(b) Mature egg treated with 300 μ M nicotine for 40 minutes. Confocal macroscopy image of the cortical actin changes (arrow) visualized with the Alexa-conjugated phalloidin and (b') merged image of the same egg. Scale bar, 50 μ m.

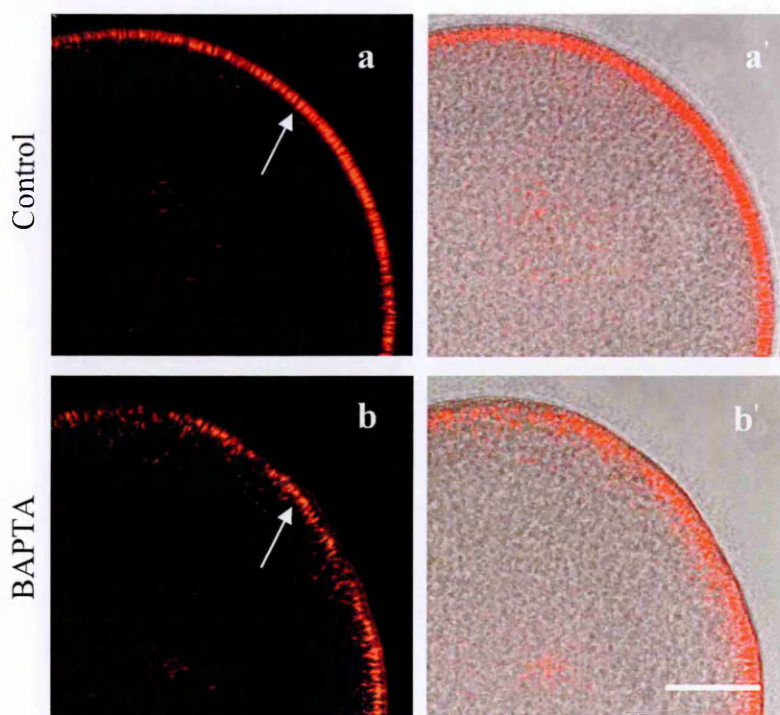


Figure V 3. BAPTA microinjection in the mature eggs causes rearrangement of the cortical actin cytoskeleton. Mature *A. aurantiacus* eggs were microinjected with Alexa-Fluor 568-conjugated phalloidin (50 μ M) and monitored with the confocal microscope 20 minutes after incubation in the seawater or, 20 minutes after the microinjection of BAPTA (100 mM, pipette concentration). **(a)** The perpendicular actin fibers located underneath the plasma membrane visualized in the control (non treated) egg with Alexa-conjugated phalloidin and **(a')** merged (transmission light and confocal) image of the same egg. **(b)** Actin fibers in the cortex are shifted inwardly 20 minutes after BAPTA-microinjection visualized with Alexa-conjugated phalloidin and **(b')** merged image of the same egg. Scale bar, 50 μ m.

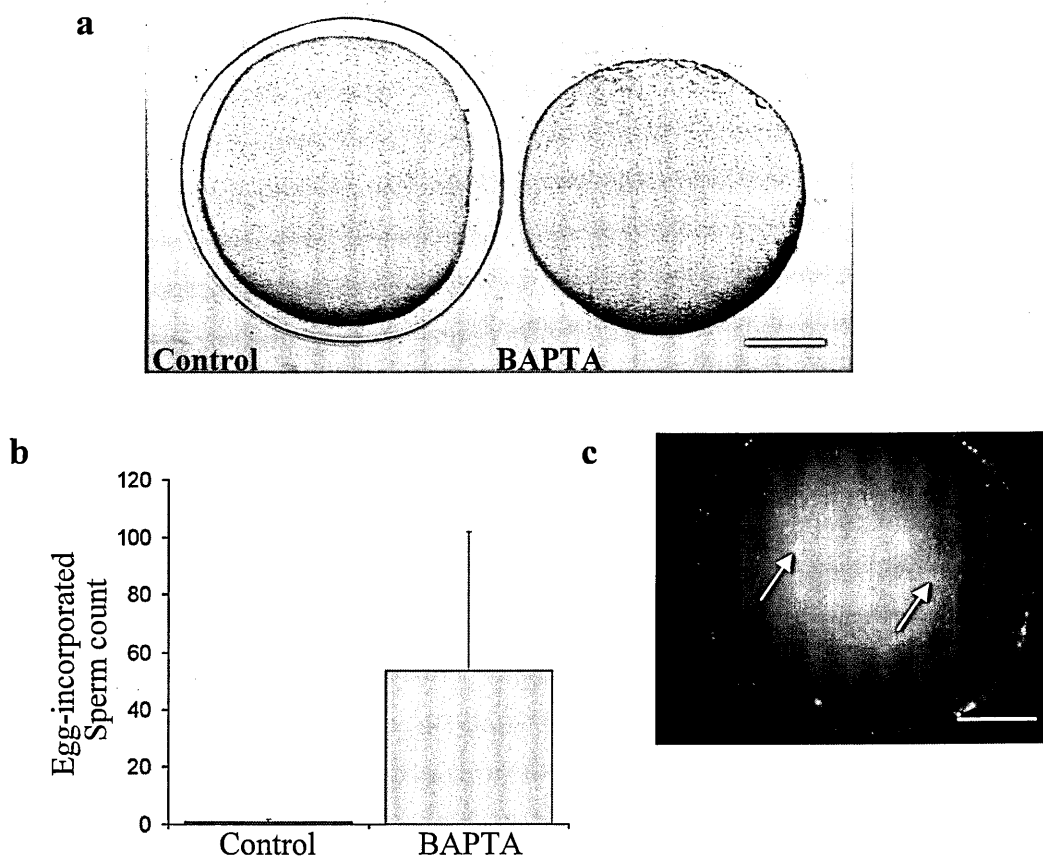


Figure V 4. Starfish eggs preinjected with BAPTA fail to elevate the vitelline layer at fertilization and lead to polyspermy. (a) Transmission light image taken with the CCD camera 15 minutes after the sperm addition to the eggs. Note the absence of the fertilization envelope in the BAPTA (100 mM, pipette concentration) preinjected egg (right egg). Scale bar, 50 μ m **(b)** Histogram of the sperm entry count in control (microinjected with injection buffer) (1.28 ± 0.56 , $n=21$) and in the eggs preinjected with BAPTA (100mM pipette concentration) (53.8 ± 48.1 , $n=16$, $P<0.0001$). **(c)** Hoechst 33342-stained sperm visualized in the BAPTA-pre-injected egg, observed on the CCD camera excited with the UV light (330 nm). Scale bar, 50 μ m.

Polyspermic fertilization may be linked to the structural features of the egg cortex at the meiotic stage

In gonads of female starfish, oocytes are arrested at the first prophase of meiosis (prophase I). These oocytes are characterized by the presence of a big nucleus termed 'germinal vesicle' (GV). Exposure of the GV stage oocytes to 1-MA leads to a resumption of the cell cycle and transition to a metaphase I. The mechanism that renders starfish eggs competent to be successfully fertilized by a single sperm is established during the so called 'maturation process' that is hallmarked by GVBD. As the oocytes ensue with the meiotic cycle, the actin cytoskeleton undergoes significant reorganization in the region subjacent to the plasma membrane. Concomitant with the actin reorganization, the apposition of the cortical granules immediately beneath the plasma membrane is essential to trigger the sperm-induced cortical granules exocytosis into the perivitelline space. By discharging the content of the cortical granules and by the vitelline layer elevation, the fertilization envelope is formed that is considered to provide a slow block against polyspermy.

The optimum period for monospermic fertilization in starfish eggs was shown to be between the GVBD and the extrusion of the first polar body (Fujimori and Hirai, 1979). Starfish eggs fertilized outside this tight time frame tend to fail to develop normally. Since this might be caused by polyspermy, I examined the number of sperm that are actually incorporated by the eggs fertilized at different meiotic stages. To do that, I added Hoechst 33342-stained sperm to the *A. aranciacus* eggs at three different stages: *i*) immature oocytes at the GV stage, *ii*) mature eggs immediately after GVBD, and *iii*) overmatured eggs 4 hours after GVBD. As expected, in the GV stage oocytes that do not undergo vitelline layer elevation when fertilized, many sperm (12.2 ± 13.7 , $n=31$) succeeded to penetrate the plasma membrane and entered the cytoplasm (Fig. V 5a and a', and Fig. V 6a). In contrast, mature eggs exposed to the same concentration of sperm displayed single sperm entry in most cases (Fig. V 5b' and Fig. V 6a). On the other hand, when overmatured eggs were fertilized, despite the full and normal elevation of the fertilization

envelope, several sperm could be seen inside the egg (Fig. V 5c and c'). While the eggs fertilized immediately after GVBD were mainly monospermic 1.12 ± 0.33 , (n=33), 4 hours post-GVBD fertilized eggs showed 3.8 ± 1.2 (n=35) sperm entered (Fig. V 6a). Hence, our data are in line with the previous report in the literature (Fujimori and Hirai, 1979), demonstrating that the frequency of a polyspermy in immature oocytes (87.1 ± 26.8 %) and overmatured eggs (100 %) is significantly higher ($P < 0.001$) than in mature eggs that have been fertilized immediately after GVBD (10.3 ± 13.1 %) (Fig. V 6b). These results of polyspermy taking place in the face of full elevation of the fertilization envelope in the overmatured eggs clearly demonstrate that the fertilization envelope formation is not the sufficient factor that warrants monospermic sperm entry in echinoderm and there might be an additional and fast mechanism that is at work to prevent supernumerary sperm entry at fertilization.

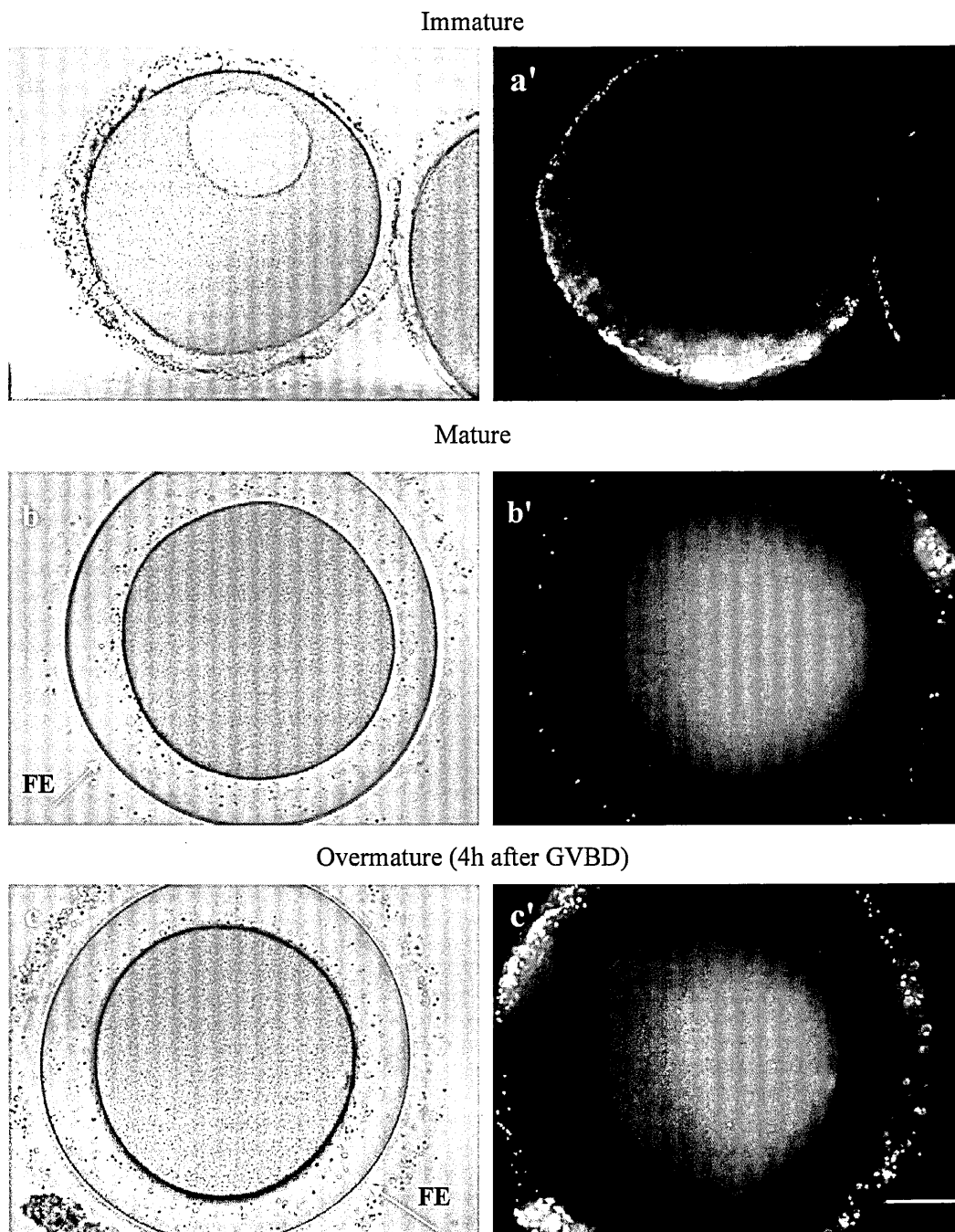


Figure V 5. Monospermic fertilization takes place only in mature eggs fertilized shortly after GVBD. (a) Transmission light image taken with the CCD camera 15 minutes after fertilization of immature oocytes, (b) mature (10 minutes after GVBD) and (c) overmatured eggs (4 hours after GVBD). (a') Polyspermic fertilization in immature oocytes. (b') Single sperm entry in mature egg. (c') Polyspermic fertilization in overmatured eggs. Sperm are visualized with the Hoechst-33342 which appears as fluorescent dots. FE - fertilization envelope. Scale bar, 50 μ m.

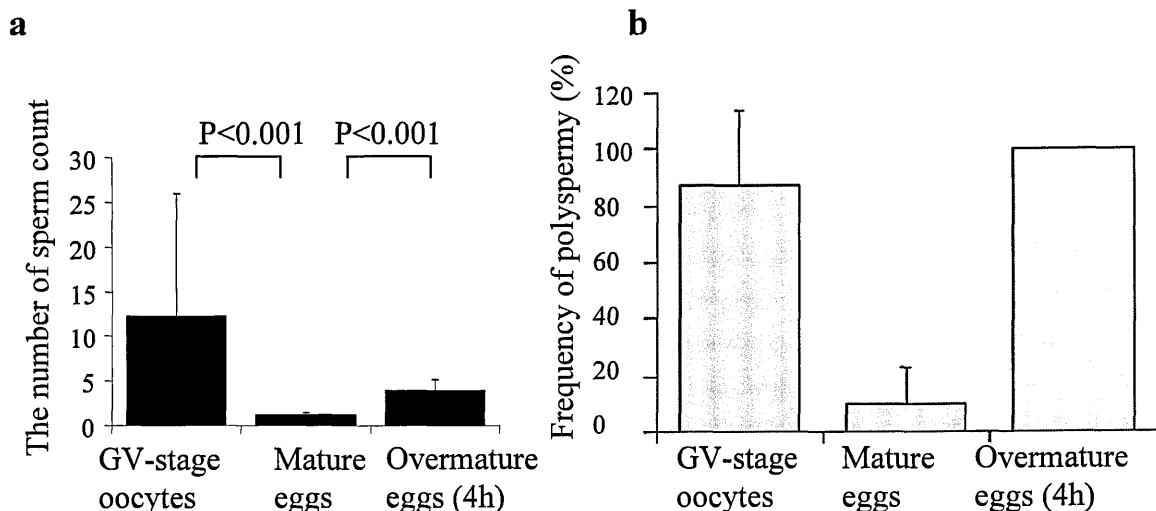


Figure V 6. High frequency of polyspermy in GV stage oocytes and overmatured (4h) fertilized eggs. Hoechst 33342-stained sperm was used to fertilize GV stage oocytes, mature eggs and overmatured (4 hours after GVBD) eggs and the sperm entry was counted on the CCD camera. **(a)** The number of sperm count in GV stage oocytes is significantly different than in mature eggs ($P < 0.001$) and the number of sperm count in overmatured eggs is statistically significant than in mature eggs ($P < 0.001$). **(b)** Frequency of polyspermy for GV stage oocytes ($87.1 \pm 26.8 \%$), mature ($10.3 \pm 13.1 \%$) and overmatured eggs (100 %).

Actin cytoskeleton as a contributing factor to the single sperm entry at fertilization

The actin cytoskeleton of the starfish oocytes and eggs undergoes finely regulated reorganization during meiotic maturation and fertilization (Lim et al., 2003; Kyojuka et al., 2008; Puppo et al., 2008). It was suggested that it may play a role in the sperm incorporation at fertilization, by facilitating the anchorage of the sperm in the fertilization cone or by establishing long microfilaments bundles at the head of the egg-incorporated sperm (Chun et al., 2010). When the structure of the actin cytoskeleton was altered with pharmacological agents and thereby its function was impaired, the number and the pattern of sperm entry were also affected (Puppo et al., 2008; Vasilev et al., 2012). If the modifications in the cytoskeleton network played a certain role in deciding this, one should expect to see similar link between the structural status of the actin cytoskeleton and the tendency of polyspermy in the eggs that are fertilized in physiological conditions as well. To test that, I have surveyed the actin cytoskeleton configuration in the starfish oocytes at different meiotic stages, which clearly displayed differential tendency to polyspermy. I have microinjected Alexa 568-conjugated phalloidin into immature oocytes, mature eggs (immediately after GVBD) and into overmatured (4h post GVBD) eggs. As shown in the confocal images (Fig. V 7), the long F-actin fibers observed in the cytoplasm of immature oocyte (Fig. V 7a) were largely absent in the mature eggs microinjected with phalloidin immediately after GVBD (Fig. V 7b). Instead, the orthogonal irregularly interconnected fine meshwork of the plasma membrane of the immature oocytes are now orderly oriented with the actin bundles perpendicularly apposed to the plasma membrane in the matured eggs. On the other hand, 4 hours after GVBD, phalloidin staining showed formation of short actin fibers in the cytoplasm and no staining in the subplasmalemmal region, which is an indication of the F-actin depolymerization (Fig. V 7c). When the phalloidin microinjected overmatured eggs was fertilized with Hoechst 33342-stained sperm and observed under the CCD camera, the presence of the incorporated sperm in the egg did not show any F-actin formation in the fertilization cones (Fig. V 8c). This was in sharp

contrast to the mature egg, in which the fertilization cone was clearly visible to be filled with actin fibers (Fig. V 8b). On the other hand, when immature oocytes were fertilized, the fertilizing cones were filled with F-actin which often took abnormal shapes, to be long and often sharply (Fig. V 8a). Hence, while the naturally occurring polyspermy in immature oocytes may be currently attributed to the lack of the fertilization elevation (no cortical vesicles exocytosis), the changes of the subplasmalemmal actin fibers in the overmatured eggs may represent a structural factor that is linked to the polyspermy that takes place in the face of the full elevation of the fertilization envelope.

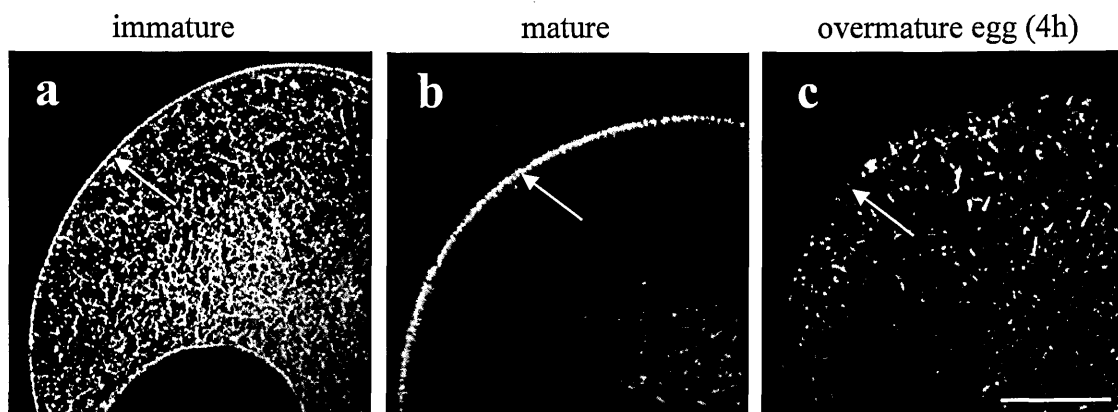


Figure V 7. Dynamic reorganization of the actin cytoskeleton in starfish oocytes during meiotic maturation. Alexa 568-conjugated phalloidin (50 μ M) was microinjected into *A. aranciacus* oocytes at diverse meiotic stages to stain F-actin in the cytoplasm. The observation was done under confocal microscope 10 minutes after incubation in natural seawater. (a) GV stage (prophase I) immature oocyte. (b) Mature egg (immediately after GVBD). (c) Overmatured (4 hours post GVBD) egg. Scale bar, 50 μ m.

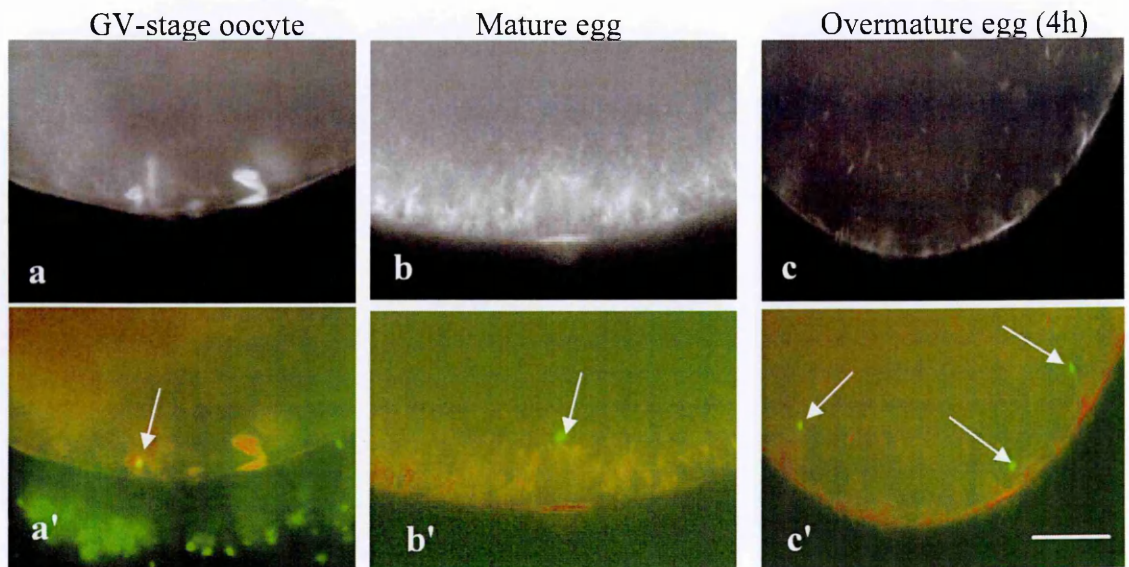


Figure V 8. Comparison of the sperm entry sites in the GV stage oocytes, mature eggs and overmatured eggs of starfish. *A. aranciacus* oocytes at the three different meiotic stages were microinjected with Alexa Fluor-568-conjugated phalloidin (50 μ M) and 10 minutes later inseminated with Hoechst 33342-stained sperm. Images taken with a CCD camera in the area of the sperm entry 5 minutes after insemination. **(a)** The actin staining in the fertilized immature oocyte and **(a')** Alexa Fluor-568 phalloidin converted to brown and Hoechst 33342 to green in order to visualize both F-actin and sperm (arrow) in the merged view of the same oocyte from **a**. **(b)** Actin fibers formation in the fertilization cone and **(b')** merged image showing the sperm entry (green dot, arrow) and the actin staining at fertilization of the same mature egg. **(c)** No actin fibers formation is visible in the site of the sperm entry in overmatured egg. **(c')** Arrows indicating the sperm entered in the overmatured fertilized egg from **c**. Scale bar, 50 μ m.

Microinjection of phalloidin in overmatured eggs alleviates polyspermy

Since the changes of the actin cytoskeleton correlate with the increased sperm entry at fertilization in overmatured eggs, shifting the polymerization/depolymerization balance of the actin network should influence the tendency toward polyspermy at fertilization in the overmatured eggs. As an initial attempt to test if the presence of the agent that stabilizes the structure of F-actin affects the number of sperm entry, overmatured oocytes were microinjected with cold phalloidin (3 mM) and incubated during the last 15 minutes of the maturation (4 hours). While the control (DMSO microinjected) overmatured eggs exhibited the expected polyspermy (3.88 ± 1.2 integrated sperm in eggs, $n=35$), the overmatured eggs with cold phalloidin (3 mM) microinjection showed significantly less number of the sperm entry (2.2 ± 2.1 , $n=26$, $P<0.05$) (Fig. V 9a). Moreover, if the obtained data are separated in a three categories, where each category represents the number of eggs fertilized by one, two or more than two sperm, while the control overmatured eggs reveal 100 % ($n=35$) polyspermy (Fig. V 9b), only 34.6% ($n=9$) of the overmatured eggs microinjected with cold phalloidin showed polyspermic fertilization, whereas others 50 % ($n=13$) are monospermic and the rest 15.4 % ($n=4$) are with no detectable sperm entry (Fig. V 9b). The data obtained indicate that the stabilization of the F-actin by cold phalloidin leads to a decreased number of sperm entry.

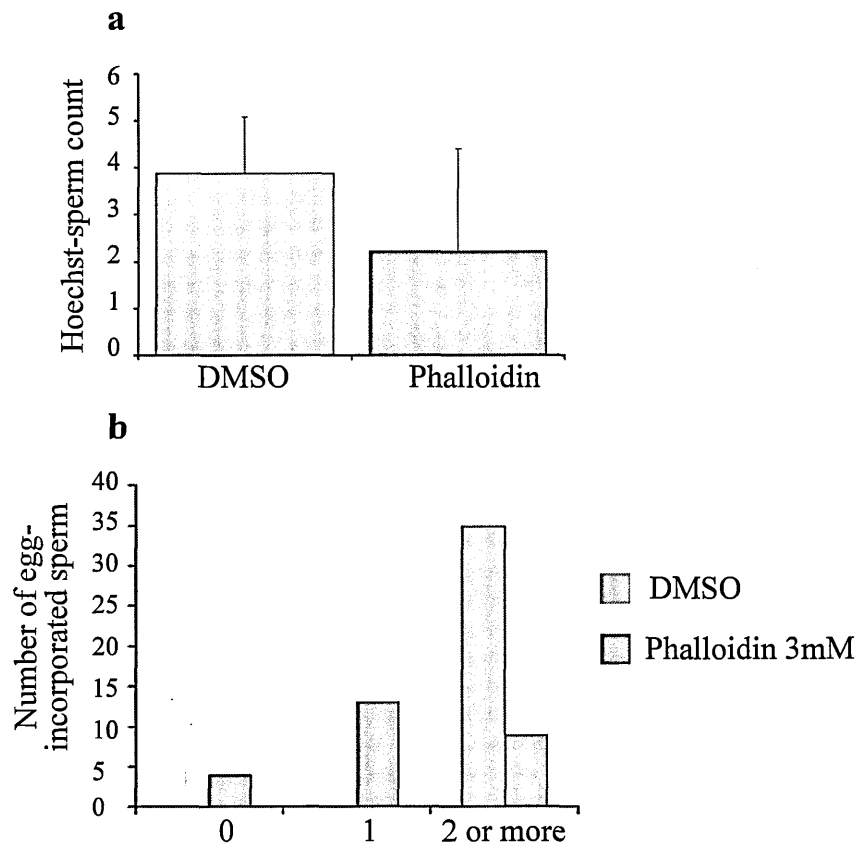


Figure V 9. Stabilization of F-actin by cold phalloidin decreases the number of the sperm entry in eggs fertilized 4h post GVBD. Sperm count in the overmatured eggs at fertilization either with DMSO (control) or with 3 mM cold phalloidin in the last 15 minutes of 4 hours incubation after GVBD. **(a)** Count of Hoechst 33342-stained sperm inside the DMSO microinjected (3.88 ± 1.2 , $n=35$) and of 3 mM cold phalloidin-injected eggs (2.23 ± 2.17 , $n=26$, $P<0.05$) eggs. **(b)** Histogram showing the results of the same experiment from **a**, indicating the number of fertilized overmatured eggs in which 0 (15.4 %, $n=4$), 1 (50 %, $n=13$) or more than 2 sperm were incorporated (34.6 %, $n=9$).

Discussion for Chapter V

The initial steps of fertilization in echinoderm eggs are characterized by plasma membrane depolarization and the sharp increase of intracellular Ca^{2+} that propagates as a wave from the sperm-egg interaction site towards the opposite pole of the egg. The intracellular Ca^{2+} rise is then followed by a cortical granules exocytosis that leads to the elevation of the vitelline layer and eventually to the formation of the fertilization envelope. In echinoderm, the formation of the thick fertilization envelope serves as a mechanism to mechanically block the entry of supernumerary sperm after fertilization. As described in the Result section, the observation of the polyspermy taking place in the face of the fully elevating fertilization envelope indicates that an additional mechanism might be normally at work before the fertilization envelope is fully elevated. That is to say, the failed block to polyspermy in the overmatured eggs (Fig. V 5) may represent the failure of the mechanism that would have otherwise prevented the entry of a few sperm in the normal and optimal egg conditions. The explanation of the underlying mechanism of the fast block to polyspermy is controversial in the literature. While demonstration of the equally fast structural changes in the egg plasma membrane is elusive, one line of thoughts have suggested that the electrical changes of the egg plasma membrane upon the successful attachment of the fertilizing sperm may be responsible for the fast block (Jaffe, 1976). Although this idea of the fast electrical block to polyspermy was corroborated in several other species (Gould-Somero et al., 1979), it was not unanimously accepted by other experimenters, who rather argued that the depolarization of the egg membrane potential might be merely the electrical consequence of the cortical granules exocytosis (Dale and Russo, 1984). Thus, the issue is still a matter of controversy in the literature (Dale and Monroy, 1981; Dale and DeFelice, 2010; Santella and Chun, 2011).

The initial work that suggested the existence of the fast block was performed on the sea urchin eggs (Jaffe, 1976). It was shown that upon the first sperm-egg contact, a small initial step depolarization across the plasma membrane takes place that precedes the further

larger depolarizing (positive) potential by several seconds. This initial sperm-induced electrical change in the egg surface was suggested to reduce the probability of additional sperm entry by a factor of 20 (Rothschild and Swann, 1950), while the later slower (about 60 seconds) change reduces the probability of a successful sperm-egg collision to zero. However, the argument that in the latent period of several seconds, before the fertilization potential reaches the highest amplitude, many sperm can bind and fuse with egg (Dale, 1985) and demonstration that holding the sea urchin eggs at negative membrane potential produces monospermic fertilization, and not polyspermic (Dale and DeFelice, 2010), cast doubts whether the fast block really exists. Finally, the discovery that the electrical fast block to polyspermy occurs in sea urchins (Jaffe, 1976) was not confirmed by others (Byrd and Collins, 1975) and in addition it was not found to occur at fertilization of mouse, hamster, or rabbit eggs (Miyazaki and Igusa, 1982; Jaffe et al., 1983; McCalloh et al., 1983).

In this chapter, it was undertaken a morphological study in order to gain more insights of the role of the actin cytoskeleton as a potential mechanism to usher the entry of single sperm in the fertilized eggs of starfish. The conventional agent used to induce polyspermy, nicotine, evoked the same effect on the fertilization of *A. aranciacus* eggs (Fig. V 1a and b). While the mechanism underlying this phenomenon has never been understood, the results of this thesis demonstrated for the first time that the nicotine produced striking alteration of the actin cytoskeleton of the eggs. Thus, in view of the previous findings that linked the actin cytoskeleton to polyspermy (Puppo 2008, Chun 2010), it is plausible that the nicotine-based polyspermy might also be attributed to the altered structure of the actin cytoskeleton in the egg cortex (Fig. V 2b). In support of the idea, the treatment of the eggs with BAPTA that produced similar changes in the F-actin structures also displayed entry of tens of sperm. This kind of failed block to polyspermy may well be attributed to the lack of the formation of the fertilization envelope that obviously serves as a mechanical block to supernumerary sperm. However, it is also

conceivable that polyspermy in these eggs treated with nicotine or BAPTA may represent a deregulated mechanism that is responsible for establishing an actin-dependent site-selective sperm interaction and entry. If so, this kind of subtle effect exerted by the alteration of the actin cytoskeleton may have been masked by the failed elevation of the fertilization envelope. At any rate, it bears an emphasis that the process of cortical granules exocytosis itself and the consequent elevation of the fertilization envelope are also dependent on the fine regulation of the actin cytoskeleton (Kyoizuka et al., 2008; Puppo et al. 2008, Chun et al., 2010; Vasilev et al., 2012).

Treatment of mature eggs for 40 minutes with 300 μ M concentration of nicotine revealed a complete blockade of the vitelline layer elevation and fertilization envelope formation. The result of this study using fluorescent F-actin probe clearly demonstrated the significant alteration of the actin cytoskeleton in the egg cortex treated by nicotine (Fig. V 2) by an unknown mechanism. The mechanism by which nicotine induces polyspermy in the eggs of another echinoderm, sea urchin, was proposed to be due to impairment (Hagstrom and Allen, 1956) or not (Longo and Anderson, 1970) of the cortical reaction, due to the alteration of the fast partial block to polyspermy (Rothschild, 1952; 1954) or due to an alteration of the gamete's surface in such a manner as to increase the probability of a successful sperm-egg collision (Rothschild and Swann, 1950; Hagstrom and Allen, 1956). As for the fast block to polyspermy that takes place in the initial seconds of the sperm-egg interaction, it was shown that nicotine can reduce the amplitude of the plasma membrane fertilization potential induced by the sperm in sea urchin (Jaffe, 1980), and thus, induce polyspermy. In these studies, nicotine seems to induce polyspermy mainly by affecting the fast block mechanism. Depending on the concentration used (from 250 μ M up to 2,5 mM in sea urchin *Arbacia punctulata*), it may or not block the vitelline layer elevation at fertilization, but still induces polyspermy (Longo and Anderson, 1970). In this thesis, 300 μ M concentration of nicotine was used to induce polyspermy and the blockage of fertilization envelope elevation. It would be interesting to see if the lower dose of nicotine

could also produce polyspermy and structural changes of the actin cytoskeleton without affecting the vitelline layer elevation. The results indicated that nicotine induces actin cytoskeleton changes, and that this structural change of the egg surface may alter the receptive field of the sperm on the egg surface. Thus, this study raises an intriguing possibility that the fine regulation of the actin cytoskeleton may serve as another factor contributing to the fast block to polyspermy. Since it was already shown that the proper actin cytoskeleton organization in the subplasmalemmal region is required for normal cortical vesicles exocytosis to occur at fertilization in starfish eggs (Puppo et al., 2008), the nicotine-induced changes in the actin, prevented the cortical reaction, and, thus, interfered also with the mechanical block to polyspermy, causing many sperm to enter the egg.

On the other hand, buffering intracellular Ca^{2+} with BAPTA induced numerous sperm entry in starfish eggs (Fig. V 4c) as it was previously shown in mouse eggs (McAvey et al., 2002). Buffering the intracellular Ca^{2+} as a result of BAPTA microinjection leads to the blockade of the cortical vesicles exocytosis and the vitelline layer elevation, which contributed to the polyspermic fertilization. Interestingly, the presence of microinjected BAPTA (100 mM pipette concentration) for 20 minutes in mature eggs, prior to fertilization, revealed very similar changes in the actin cytoskeleton structure, as observed in the nicotine treated eggs (Fig. V 2b), by showing actin polymerization in the cortex shifted towards the inner cytoplasm (Fig. V 3b). The actin cytoskeleton perturbations evoked by microinjection of BAPTA has already been described to take place in various cell types. Significant depolymerizing effect on the actin cytoskeleton has been shown for mammalian cells such as MDCK cells, mouse myoblasts and for A6 *Xenopus* cell line (Saoudi et al., 2004). At least in the *Xenopus* cells the effect on the actin cytoskeleton disassembly was demonstrated to be independent of the Ca^{2+} chelation activity (Saoudi et al., 2004). Nevertheless, nothing more is known about how BAPTA induces changes in the actin cytoskeleton. Interestingly, in the same study (Saoudi et al., 2004), it was also shown that the effect on the cytoskeleton in the BAPTA-AM

incubated RAT2 cells was reversible, displaying actin assemblies re-formed within 2 hours after the removal of the Ca^{2+} chelator from the medium.

During the transition from the immature (GV stage) oocyte to the mature egg induced by the maturation hormone 1-MA, there is a dramatic reorganization in the actin cytoskeleton structure which is important for the sensitization of the intracellular Ca^{2+} stores and precise cortical granules orientation beneath the plasma membrane (Chiba et al., 1990; Longo et al. 1995; Santella et. al., 1999; Lim et al., 2003; Hirohashi et al, 2008). Immediately after the GVBD has occurred, matured eggs acquire the capacity to be successfully fertilized by one sperm, again with the active contribution of the actin cytoskeleton, which is thought to play important roles in establishing the sperm-egg contact and enabling the cortical vesicles exocytosis and the sperm entry (Puppo et al., 2008; Chun et al., 2010; Vasilev et al., 2012). However, when *Asterias amurensis* mature eggs are fertilized 3 hours after GVBD (overmatured), they display polyspermy and abnormal development (Fujimori, 1979). This data indicates the existence of the optimal window time for the successful monospermic fertilization to occur, which is before the first polar body extrusion. In line with the idea that the occurrence of polyspermy is linked to the meiotic stage of the eggs, the data of fertilized *A. aranciacus* overmatured eggs (Fig. V 6a) have confirmed the results of the other starfish specie (*Asterias amurensis*). In addition, the actin cytoskeleton structure in these overmatured eggs (Fig. V 7c), revealed a significant changes in its structure as compared to the mature monospermic eggs (Fig. V 7b). The apparent actin depolymerization in the cortex could affect the structure of microvilli which in turn may affect the normal sperm-egg interaction (Fig. V 7c). Being important for the sperm anchorage and its incorporation into the egg cytoplasm, the absence of the actin polymerization in the fertilization cone of the fertilized overmatured egg (Fig. V 8c) presumably made the egg surface easily penetrable for the sperm (Fig. V 8c', note 3 sperm entered). Starfish eggs susceptibility to polyspermy increases with the time after spawning, and this phenomenon was correlated with the time-dependent

decrease in the fertilization potential amplitude (Miyazaki and Hirai, 1979). In this thesis, 94 % (33 out of 35) of overmatured eggs successfully undergoing fertilization envelope formation displayed 100 % polyspermy (Fig. V 6b), and these striking results concomitantly took place with the drastic structural alteration of the actin cytoskeleton at the plasma membrane. Thus, the result of this thesis suggests that the proper readiness of the actin cytoskeleton in the egg surface might also be linked to the establishment of a fast block for polyspermy in starfish eggs at fertilization.

During fertilization of starfish eggs, it takes up to 3 minutes for the vitelline layer elevation and hardening of the consequent fertilization envelope, which appears to be a mechanical block to the entry of supernumerary sperm. If we assume that the fast block does not work, then what prevents the additional sperm entry in this window time? An idea was put forward, suggesting that the successful sperm entry happens in the strictly defined sites on the egg surface and these sites are rich with F-actin (Dale and DeFelice, 2010). In favour of this idea was shown that when immature oocytes of starfish (Fig. V 6a) and sea urchin are fertilized, only 10-15 sperm can penetrate the oocytes. Even if it is assumed that all sperm were fertilization-competent and get in close contact with the immature sea urchin oocytes, only these small number of sperm penetrate the oocyte (Dale, 1985). On the other hand, it is well known that it is not the first sperm that arrives to the mature egg, either sea urchin or starfish, that establishes the strong interaction and consequently to enter the egg. In this context, the data in this thesis are strongly supportive of the idea that the actin cytoskeleton may contribute to regulate the number of this cryptic sperm receptive sites. As shown previously, microfilaments-filled microvilli may play a role in gamete fusion (Carron and Longo, 1982; Sato and Osani, 1986). During meiotic maturation their number strongly reduces and by the end of the GVBD their shape becomes shortened (Hirai and Kanatani, 1973; Cayer et al., 1975). Hence, it is possible that there is a progressive decrease in the number of sperm-engaging sites. It is tempting to speculate that in the initial minutes of fertilization, upon the first sperm has been

successfully anchored to the plasma membrane on the microvilli, the other microvilli on the egg surface could retract so that no other sperm could interact with the egg. However, sometimes even mature eggs are polyspermic, and can be made polyspermic with various chemical agents. Inducing polyspermy with nicotine and BAPTA as it was shown in the results section, interfere with the normal cytoskeleton structure (Fig. V 2 and 3). Further, the actin polymerization during the extension of the fertilization cone (Fig. V 8b), formation of the thick actin bundles in the site of the sperm entry (Chun et al., 2010) and the cortical fibers migration (Terasaki, 1996; Vasilev et al., 2012) during the sperm entry, again underline the important morphological changes driven by actin cytoskeleton at fertilization. Shifting the fertilization process toward mono or polyspermy by altering the actin cytoskeleton, might indicate that the sperm-transmitted information to the egg might cause swift changes in the cortical actin that could further interfere with the additional sperm-egg interactions.

Stabilizing the actin cytoskeleton by microinjecting a high concentration of cold phalloidin, reduced the number of the sperm entry in overmatured eggs (Fig. V 9a). Moreover, it produced considerable number of eggs in which none of the sperm entered (15 %), or in which only one sperm was incorporated (50 %). This numbers, again, confirm the important role the actin cytoskeleton plays at fertilization. Firstly, the fact that there are eggs that do not have any sperm incorporated, indicates that the stabilized actin structures in the microvilli heavily affected the sperm-egg interaction, presumably, due to disturbances of the correct microvilli structure unable to establish strong contact with the sperm. Alternatively, in the case if the normal interaction was established, by blocking the long actin microfilaments in the site of the sperm entry and newly formed F-actin during cortical fibers migration at fertilization, it could have interfered with the sperm incorporation into the egg. However, the stabilizing effect on the actin cytoskeleton in 50 % of the same batch of the cold phalloidin microinjected eggs, seemed to return the lost ability of overmatured eggs to be fertilized by one sperm, presumably due the actin

microfilaments stabilization itself, or to an unknown factors that apparently are dependent on the dynamic state of the actin. The most resistant 35 % of the overmatured eggs managed to overcome the stabilizing effect, and showed slight decrease in the sperm entry number, still being polyspermic as control eggs. The finding that the alteration in the actin networks in the eggs, causes abnormality in the fertilization process was already shown for the ionomycin pretreated starfish eggs (Vasilev et al., 2012) and for the mouse eggs, that have undergone changes in the acto-myosin structure (Matson et al., 2006; Larson, 2009). Since the receptor proteins residing on the plasma membrane on the microvilli of mammalian eggs (Runge et al., 2007), that have been shown to be a binding partners for the receptors on the sperm plasma membrane in the process of sperm anchorage to the egg, it is also conceivable that these receptors interact with the actin cytoskeleton. If analogous takes place in starfish eggs, then, altering the actin cytoskeleton could lead to their structural changes which may affect their function, which in turn might lead to the failure of gametes recognition and fusion.

Conclusions

The major findings in the present thesis are:

1. The fine regulation of the actin filaments is essential for the sensitization of the InsP_3 -inducing Ca^{2+} release during meiotic maturation because the depactin antibody microinjected into oocytes reduces the InsP_3 -mediated Ca^{2+} signals in mature starfish eggs.
2. Sequestration of the PIP_2 during meiotic maturation of starfish oocytes impeded the translocation of the cortical granules that normally align in the tight space beneath the plasma membrane.
3. Ionomycin induces intracellular Ca^{2+} increase from the internal stores and from Ca^{2+} influx and the pretreated oocytes displayed a compromised Ca^{2+} response to InsP_3 and sperm, and no response to the second dose of ionomycin.
4. Ionomycin induces significant alterations in the structure of the actin cytoskeleton and in the structure of the cortical vesicles.
5. Ionomycin-pretreated eggs often display unsuccessful sperm entry at fertilization, and the resulting zygotes, even the monospermic ones, failed to develop normally.
6. Stabilization of the actin cytoskeleton interferes with a successful sperm entry and reduces the rate of polyspermy in overmatured starfish eggs.
7. Nicotine and BAPTA dislodge the subplasmalemmal actin fibres in a strikingly similar pattern, and this may be linked to their capability of inducing polyspermy.

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